

Mechanism of Regulation of Protein Kinase Aurora A in Response to Mitotic DNA Damage

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DEDICATION

This thesis is dedicated to my grandmother, Biji.

I was lucky to see you before you left us forever.

You will always be missed.

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ZUSAMMENFASSUNG

Die Mitose ist ein hochgradig geordneter Prozeß der sicher stellt daß das duplizierte Genom gleichmäßig auf die Tochterzellen aufgeteilt wird. Gelingt dies nicht, so geht genetische Information verloren was zu Aneuploidy führt, wie es oft in Krebszellen beobachtet werden kann. Mehrere mitotische Kinasen sind das Ziel des sogenannten „Checkpoint“, welcher DNS Schäden detektiert. Die wichtigsten Kinasen hierbei sind Cdk1, Aurora A und Plk1. Aurora A, welche im Mittelpunkt dieser Studie steht, wird durch Schäden an der DNS während der G2 Phase inaktiviert. Dies führt zu einem ATM/ATR-Chk1 abhängigen Zellzyklus Arrest.

In dieser Studie werden die molekularen Mechanismen untersucht, welche zu DNS Schadens-bedingter Inhibition von Aurora A Aktivität führen. Wir konnten auch zeigen, daß Aurora A, ein Ziel der IR-induzierten DNS Schäden, während der Mitose ist. Wir haben einen Synchronisationsansatz benutzt welcher die Zellen in der Mitose blockiert, während welcher die Aurora A Kinase Aktivität am höchsten ist. Als Maß für die Aktivität, haben wir die Phosphorylierung von Aurora A an der „T-loop“ Aminosäure, T288 getestet, indem wir einen spezifischen Antikörper benutzt haben. Das Resultat bestätigt ein reduziertes Phospho-Signal, welches Indikativ ist für die reduzierte Kinase Aktivität. Indem wir den Verlust der Phosphorylierung an T320 gemessen haben, konnten wir bestätigen daß Protein Phosphatase 1 (PP1) aktiviert wurde durch mitotische DNS Schäden. Diese ist eine Cdk1 abhängige Phosphorylierungsstelle in PP1. Dies deutet an, daß PP1 die Phosphatase ist welche für die Aurora A T228 dephosphorylierung verantwortlich ist.

Während der Mitose ist TPX2, ein Mikrotubulus assoziiertes Protein, der Hauptregulator von Aurora A. TPX2 bindet Aurora A und erleichtert dessen Lokalisierung an den mitotischen Spindelapparat und zusätzlich aktiviert es Aurora A indem es T228 vor einer dephosphorylierung durch PP1 bewahrt. Diese Interaktion sorgt dafür daß die Kinase während der Mitose in einer aktiven Konformation bleibt. Wir haben eine Inaktivierung von Aurora A durch mitotischen IR Schaden beobachtet, welche mit dem auseinanderfallen des Aurora A-TPX2 Komplexes korreliert. Dies wiederum ist das Resultat eines verminderten TPX2 Protein Niveaus.

Indem wir Cycloheximid benutzt haben um die Protein Synthese zu inhibieren konnten wir feststellen daß als Antwort auf mitotische DNS Schäden, TPX2 sehr instabil wurde. TPX2 wird durch die APC-Cdh1 Proteasom Kaskade degradiert. Wir konnten zeigen, daß eine Reduktion der TPX2 Gesamtpopulation signifikant durch Post-translationale Kontrollmechanismen beeinflußt wird, da das TPX2 mRNA Niveau durch DNS Schäden unbeeinflußt blieb. Ein erster Versuch, diese Kaskaden zu identifizieren deutet einen Defekt während dem Prozeß der Translationsinitiation an. Dies wird reflektiert durch die Reduzierte Menge an TPX2 welche mit den aktiv translatierenden Einheiten, den Polysomen Assoziiert.

Zusammengefaßt deuten unsere Resultate darauf hin, daß mitotische DNS Schäden die Instabilität von TPX2 erhöht. Dadurch daß weniger TPX2 neu synthetisiert wird, kommt es zu einem Ungleichgewicht des existierenden Protein Haushalts. Dies beeinflußt den Aurora A-TPX2 Komplex, was wiederum dazu führt daß die pT288 Phosphorylierungsstelle von aktivem PP1 angegriffen wird und somit Aurora A inaktiviert.

SUMMARY

Mitosis is a highly ordered collection of events that ensures that the duplicated genome is distributed to the daughter cells equally. A failure to do so results in loss of genetic information leading to aneuploidy, a condition frequently associated with cancer. Several mitotic kinases are targets of the DNA damage checkpoint, among which Cdk1, Aurora A and Plk1 are the most significant. Aurora A, the focus of study presented in this thesis, was shown to be inactivated by DNA damage induced in the G2 phase, leading to cell cycle arrest in an ATM/ATR-Chk1 dependent manner.

In the present study, we addressed the molecular mechanism leading to DNA damage-induced inhibition of Aurora A activity. We show that Aurora A is also a target of IR-induced DNA damage occurring in mitosis. We used a synchronization approach that arrests cells in mitosis where Aurora A kinase activity is at the peak. As a measure of its activity, we tested the phosphorylation of Aurora A at the T-loop residue, T288, using specific antibody. The results confirmed a decreased phospho-signal, indicative of reduced kinase activity. We confirmed that protein phosphatase 1 (PP1) was activated by mitotic DNA damage by scoring the loss of phosphorylation at T320, a Cdk1-dependent phosphosite in PP1, indicating with high probability that this is the phosphatase responsible for Aurora A T288 dephosphorylation.

During mitosis, TPX2, a microtubule-associated protein, is the main regulator of Aurora A. TPX2 binds to Aurora A facilitating its localization to mitotic spindles and, in addition, activates it by protecting T288 from PP1-mediated dephosphorylation. This interaction ensures that the kinase is locked in an active conformation throughout mitosis. Upon IR-induced mitotic damage, we observed inactivation of Aurora A in a manner that was directly linked to disruption of the Aurora A-TPX2 complex. This, in turn, was the result of decreased TPX2 protein level. By employing cycloheximide to prevent any nascent protein synthesis, we found that in response to mitotic DNA damage, TPX2 became highly unstable, being degraded by the APC-Cdh1 proteasome pathway with faster kinetic than in control cells. We showed that decrease of the overall population of TPX2 was also significantly contributed by post-transcriptional control mechanisms, as the TPX2 mRNA level remained unvaried in the presence of damage. An initial attempt to identify these pathways indicated a

defect in the process of translation initiation, as seen by the reduced level of TPX2 mRNA that was able to associate with the actively translating units, the polysomes.

Collectively, our results indicate that upon mitotic DNA damage, increased TPX2 protein instability and, particularly, lack of new TPX2 synthesis results in an overall unbalance of the existing protein pool in a manner that affects the Aurora A-TPX2 complex. This, in turn, exposes the pT288 site to activated PP1 resulting in inactivation of Aurora A.

1. INTRODUCTION

1.1. PROTEIN KINASES AND PHOSPHORYLATION

Phosphorylation is the most prevalent post-translational modification that regulates several processes such as signal transduction, cellular metabolism, growth and differentiation, cell cycle transitions and apoptosis. The effect is mediated by modifying the structure, localization, function and stability of the phosphorylated proteins. Enzymatically, this is mediated by class of enzymes called protein kinases (PKs). 2% of the human genes encode for PKs, classified majorly as eukaryotic PKs (ePKs), making them one of the largest families of genes in eukaryotes (Ubersax and Ferrell, 2007). There are, however, also some atypical PKs (aPKs) that although possess kinase activity, do not have any structural homology to the ePK catalytic domain (Manning et al., 2002).

The basic mechanism of action of PKs involves transfer of the phosphate group from donors such as ATP or GTP to a serine (ser), threonine (thr) or tyrosine (tyr) residue in the substrates (Fig. 1). Once the substrate is phosphorylated, it is released from the kinase and ADP/GDP is recycled.

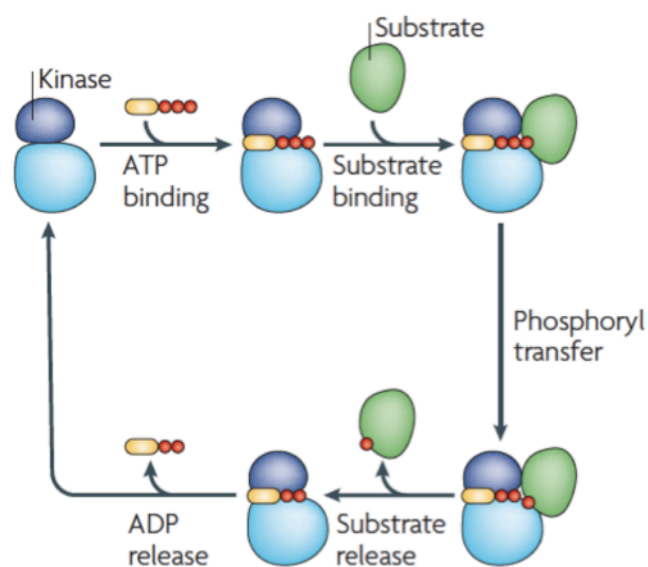


Figure 1- Mechanism of action of a protein kinase. (See text for explanation). (Adapted from Ubersax and Ferrel, 2007)

Structurally, protein kinases are similar owing to the presence of a catalytic domain that consists of a small N-terminal lobe of β -sheets and a large C-terminal lobe of α -

helices (Ubersax and Ferrell, 2007). Together, the catalytic domain is made up of 12 sub-domains encompassing the two lobes (Fig. 2) (Ferrari, 2006). The cleft between the two lobes holds the ATP. The subdomain I contains a glycine-rich motif called the P-loop, while a region called the activation segment is present in sub-domains VII and VIII (Ferrari, 2006). The activation segment contains one or two phosphorylation sites that regulate activation of many kinases (Johnson and Lewis, 2001). The activation segment consists of a Mg^{++} -binding loop that chelates the Mg^{++} -ATP pool, an activation loop that has the regulatory phosphorylation residues and a P+1 loop that facilitates the reaction by substrate binding (Ferrari, 2006).

Specificity of a protein kinase for its substrates is a complex matching arrangement. Factors such as depth of the active site and domains present in the adjacent docking sites in the kinase, presence of targeting subunits and localization of the kinase play extremely important roles in determining substrate-specificity (Ubersax and Ferrell, 2007). Examples of such regulatory events and how they contribute to the activity of the kinases is described in more details in the following sections.

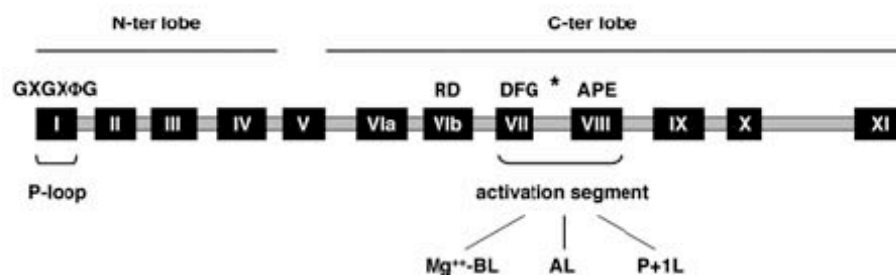


Figure 2- Structure of the catalytic domain of PKs. The 12 sub-domains that constitute the N- and C-terminal lobe of the catalytic domain of a protein kinase are shown. The asterisk represents the position of residues in the activation segment that activate the kinase upon being phosphorylated. Mg^{++} -BL, Mg^{++} -binding loop; AL, activation loop; P+1L, P+1 loop. (Adapted from Ferrari S, 2006)

1.2. CELL CYCLE

The eukaryotic cell cycle can be defined as a highly regulated series of events that lead to eukaryotic cell division. This essentially consists of the **S (Synthesis)** phase that allows duplication of chromosomes via DNA replication followed by the **M (Mitotic)** phase that monitors equal distribution of the duplicated genome to daughter cells. These two phases are separated by gap (or growth) phases, G1 and G2. In G1 phase important decisions are made to either commit to division or to exit from the cell cycle. The G1 and G2 phases also provide additional time for the cell to prepare for growth and host control mechanisms that allow progression to the next phase only under optimal conditions (both intrinsic and extrinsic to the cell). The main players of the eukaryotic cell division cycle are the **Cyclin-dependent kinases** that are activated by their binding partners **cyclins** and regulate every event of cell cycle progression.

Here, I review in detail the mechanism of activation of Cyclin-dependent kinases by cyclins.

1.2.1. CYCLIN-DEPENDENT KINASES (Cdks)

Cdks constitute a highly conserved family of ser/thr protein kinases that are the central components of eukaryotic cell cycle. Structurally, they consist of a catalytic subunit and a regulatory subunit called cyclin; binding of the latter stimulates the catalytic activity of Cdks. The concentration of Cdks remain constant throughout the cell cycle while that of cyclins vary dramatically (Morgan, 1997). This ensures that only Cdks specific to the ongoing phase of the cell cycle will be activated. In yeast, a single Cdk (Cdc28 in *S.cerevisiae* and Cdc2 in *S.pombe*) regulates all cell cycle events while mammalian cells have several Cdks specific to different phases (Table I) (De Bondt et al., 1993).

1.2.1.1. Activation of Cdk by Cyclin

Protein kinases have a common tertiary structure consisting of a small N-terminal lobe and a large C-terminal lobe within the catalytic domain. A deep cleft between

these two lobes forms the site of ATP-binding (Ferrari, 2006). In the absence of cyclins, Cdks have an unfavorable tertiary structure that on the one hand impairs substrate binding and on the other hand does not allow correct positioning of the phosphates of ATP. Therefore, in order to be able to carry out the enzymatic reaction, Cdks require major structural changes that are facilitated by binding of cyclins.

Cyclins interact directly with the PSTAIRE helix in the Cdk N-terminal lobe resulting in reorientation of the residues that interact with the phosphates of ATP and facilitate the phosphotransfer reaction. The active site also undergoes a major change and forms β -strand upon cyclin binding (De Bondt et al., 1993). This allows substrate binding and therefore facilitates phosphorylation by Cdks (Morgan DO, *The Cell Cycle: Principles of Control*). Phosphorylation by Cdk-activating kinase (CAK) of a highly conserved thr residue, T160, in Cdk2 is also favored in such a conformation and this, in turn, strengthens the cyclin-Cdk interaction.

1.2.1.2. Functions of Cdk associated cyclins

Apart from binding and activating Cdks, cyclins play important functions throughout the cell cycle. One such function is to spatially and temporally regulate the sub-cellular localization of the Cdks. Cyclin B exists in two forms, cyclin B1 and B2. Before the onset of mitosis, the cyclin B1/Cdk1 complex is largely cytosolic. In late prophase, however, it is rapidly translocated to the nucleus where it phosphorylates the nuclear lamins to initiate nuclear envelope breakdown (NEBD). Although cyclin B1 does not possess any nuclear localization signal (NLS), multiple phosphorylations at its N-terminal region govern its nuclear accumulation (Morgan DO, *The Cell Cycle: Principles of Control*). Cyclin B2 associates with the Golgi apparatus and Cdk-dependent phosphorylations of proteins mediate fragmentation of the organelle during mitosis. In yeast, the S-phase cyclin Clb5 binds to the origins of replication regulating S-phase progression (Morgan DO, *The Cell Cycle: Principles of Control*). Clb5 also interacts with several proteins involved in DNA replication. Cyclin A/Cdk but not cyclin B/Cdk binds and phosphorylates p107 to regulate G1-S progression (Morgan DO, *The Cell Cycle: Principles of Control*). Cyclins, therefore, regulate Cdk functions also by directly interacting with their substrates.

A description of the cyclin/Cdk complexes of eukaryotic cell cycle is shown below.

A					B				
Cyclin-Dependent Kinases					Cyclin class (with Cdk partner)				
Species	Name	Original name	Size (amino acids)	Function	Species	G1	G1/S	S	M
<i>S. cerevisiae</i>	Cdk1	Cdc28	298	all cell-cycle stages	<i>S. cerevisiae</i>	Cln3 (Cdk1)	Cln1,2 (Cdk1)	Clb5,6 (Cdk1)	Clb1,2,3,4 (Cdk1)
<i>S. pombe</i>	Cdk1	Cdc2	297	all cell-cycle stages	<i>S. pombe</i>	Puc1? (Cdk1)	Puc1, Cig1? (Cdk1)	Cig2, Cig1? (Cdk1)	Cdc13 (Cdk1)
<i>D. melanogaster</i>	Cdk1	Cdc2	297	M	<i>D. melanogaster</i>	cyclin D (Cdk4)	cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B, B3 (Cdk1)
	Cdk2	Cdc2c	314	G1/S, S, possibly M					
	Cdk4	Cdk4/6	317	G1 promotes growth					
<i>X. laevis</i>	Cdk1	Cdc2	301	M	<i>X. laevis</i>		cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B (Cdk1)
	Cdk2		297	S, possibly M	<i>H. sapiens</i>	cyclin D1,2,3 (Cdk4,6)	cyclin E (Cdk2)	cyclin A (Cdk2,1)	cyclin B (Cdk1)
<i>H. sapiens</i>	Cdk1	Cdc2	297	M					
	Cdk2		298	G1/S, S, possibly M					
	Cdk4		303	G1					
	Cdk6		326	G1					

Table I- Description of A) Cdk and B) Cyclins that control the eukaryotic cell cycle. (Adapted from Morgan DO, *The Cell Cycle: Principles of Control*)

In this section, I review each phase of the cell cycle in detail.

1.2.2. G1-S transition

As mentioned above, different cyclin/Cdk complexes drive the cell cycle and specific cyclin/Cdk complexes regulate entry into G1 and G1 to S transition. The major G1 cyclins are cyclin D and E. Early cell cycle phases are dependent on transcription factors of the E2F family that include both repressors (E2F-4,5) and activators (E2F-1-3). In G0 or before the 'Start', transcription of genes required for entry into the cell cycle is largely suppressed by E2F transcription repressors that are bound by pocket proteins, pRb (retinoblastoma), p130 and p107 (Cam and Dynlacht, 2003) (Morgan DO, *The Cell Cycle: Principles of Control*). Activator E2Fs are present in very small amounts in quiescent cells and their transcription is also repressed by E2F repressors (Morgan DO, *The Cell Cycle: Principles of Control*). In G0, pRb is

hypophosphorylated and active; this results in inhibition of transcription of genes (required for progression of G1) by the E2F transcription factor. This inhibition is mediated via two distinct mechanisms (Fig. 3): pRb can directly bind to the transactivation domain of E2F or it can recruit chromatin-remodeling complexes to the promoter region, preventing transcription (Harbour and Dean, 2000). pRb can simultaneously bind to histone deacetylases (HDACs) and E2F thereby promoting deacetylation of the promoters and transcription silencing.

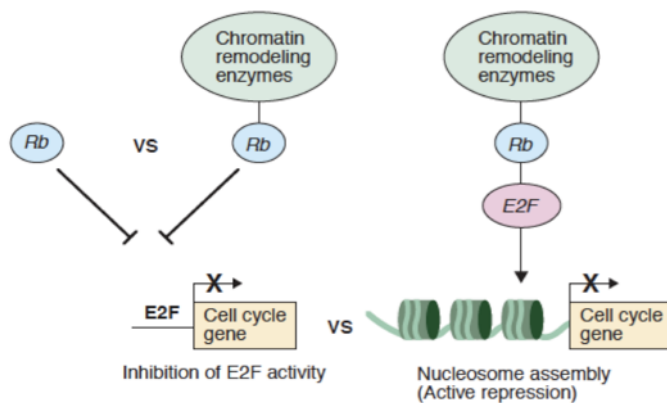


Figure 3- E2F-dependent transcription inhibition by Rb protein. Rb protein can either bind directly to the E2F transcription factor or recruit chromatin-remodeling enzymes that alter the chromatin resulting in transcription

repression. (Adapted from Harbour and Dean, 2000)

In response to growth factor stimulation (mitogens) in G0, there occurs an increased synthesis of the D-type cyclins through Ras and Myc activated signaling cascades, resulting in their assembly with Cdk4/6. This is further facilitated by decreased amounts of Cdk inhibitors of the Cip/Kip family (Olashaw and Pledger, 2002). Active cyclin D/Cdk4/6 complexes regulate G1 progression through phosphorylation of pRb (Fig. 4). Phosphorylation of pRb first occurs by the cyclin D/Cdk4/6 complex that allows transcription of genes required for G1 phase, sequesters the Cip/Kip proteins and facilitates activation of cyclin E/Cdk2 complex. Further phosphorylation of pRb by cyclin E/Cdk2 is required for complete inactivation of pRb and re-activation of transcription (Harbour and Dean, 2000). This full activation of transcription sets the stage for S-phase entry.

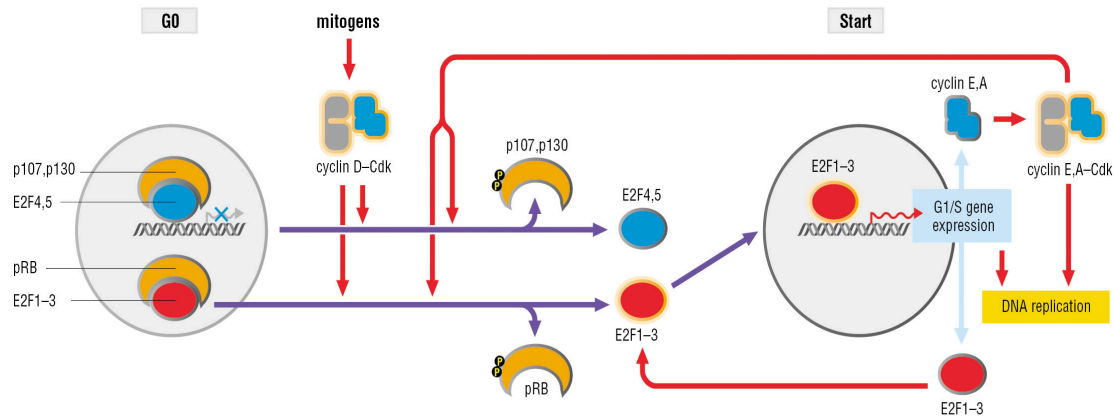


Figure 4- Control of E2F-dependent gene expression during entry into the cell cycle. In G0, E2F-dependent transcription of G1-S genes is repressed by interaction with the pRb protein. Mitogen activation of the cyclin D/Cdk complex results in phosphorylation of pRb, dissociating it from E2F and promotes expression of cyclin E and A. The cyclins then participate in a positive feedback loop to further activate E2F. (Adapted from Morgan DO, *The Cell Cycle: Principles of Control*)

1.2.3. S (Synthesis) phase

Inhibition of pRb activity by cyclin D/Cdk4/6-mediated phosphorylation initiates entry into S-phase. Cyclin A/Cdk2 is the major driver of S-phase that is activated by Cdc25A-dependent dephosphorylation (Morgan DO, *The Cell Cycle: Principles of Control*). Accumulation of cyclin A in late G1 depends on its increased expression by E2F mediated transcription. The derepressed and active E2F transcription factor forms heterodimeric complex with the DP-1 protein and promotes transcription of genes required for S-phase (Fig. 5) (Shapiro, 2006). This is, however, only an initial and transient E2F activation. Multiple phosphorylations by S-phase specific Cdks result in shutting off of the E2F activity later in S-phase. Cyclin A/Cdk2 interacts with the NTD of E2F and phosphorylates S307 that inhibits the E2F-DP-1 DNA binding activity (Peeper et al., 1995). Cyclin A/Cdk1 phosphorylates E2F at S375 resulting in formation of the pRb-E2F complex promoting inactivation of E2F mediated transcription (Peeper et al., 1995). In addition, cyclin H/Cdk7, the Cdk associated with RNA polymerase II, phosphorylates E2F-1 at S408 and T433, which promotes

its ubiquitination-mediated degradation (Vandel and Kouzarides, 1999). Decreased expression of cyclin A thus initiates the inactivation of the cyclin A/Cdk2 complex, facilitating progression to the next phase of the cell cycle.

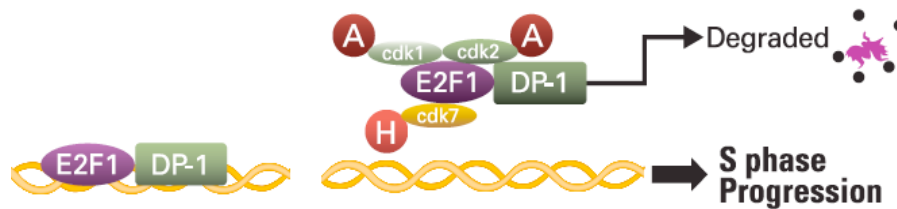


Figure 5- E2F-dependent S-phase progression. (See text for explanation). (Adapted from Shapiro, 2006)

1.2.4. G2-M transition

In higher eukaryotes, entry into mitosis is primarily regulated by cyclin A and B and Cdk1. Cyclin B1/Cdk1 was initially identified as the maturation-promoting factor (MPF), a cytoplasmic activity capable of facilitating meiosis I and II in the absence of any hormonal stimulation in *Xenopus* oocytes (Duesbery and Vande Woude, 1998). This was later also found in mitotic cells (Lohka et al., 1988) and therefore was also called the mitosis-promoting factor. The cyclin B1 protein level rises during G2 and this allows its interaction with Cdk1 in the cytoplasm. The cyclin B1/Cdk1 complex is kept inactive in the cytoplasm by phosphorylation at T14 and Y15 residues by protein kinase Myt1 (membrane-bound) and Wee1 (soluble and nuclear) (Liu et al., 1997). Different localization of the two inhibitory kinases ensures complete inactivation of different sub-populations of Cdk1.

Phosphorylation of Y15 results in blocking of transfer of the phosphate group to the bound substrate (Atherton-Fessler et al., 1993), while T14 phosphorylation interferes with binding of ATP (Endicott et al., 1994). Once the complex is transported to the nucleus, CAK phosphorylates Cdk1 at T161, partially activating the kinase. This results in phosphorylation and activation of Cdc25 family of phosphatases by Cdk1,

setting a positive feedback loop for its own activation. Cdc25 phosphatases counteract the inhibitory phosphorylations on Cdk1, further activating it. Initial activation of Cdk1 starts in late G2 by dephosphorylation mediated by Cdc25B that is active in G2. As the cells enter prophase, the protein level of Cdc25A and catalytic activity of Cdc25C rises dramatically, leading to increased Cdk1 activation (Izumi and Maller, 1993), (Lindqvist et al., 2005).

Another mitotic kinase, Plk1, plays an important role in activation of Cdk1. Partially active Cdk1 results in Plk1 activation that allows the latter to phosphorylate and activate Cdc25C and inhibit Myt1, facilitating Cdk1 activation (Fig. 6) (Barr et al., 2004).

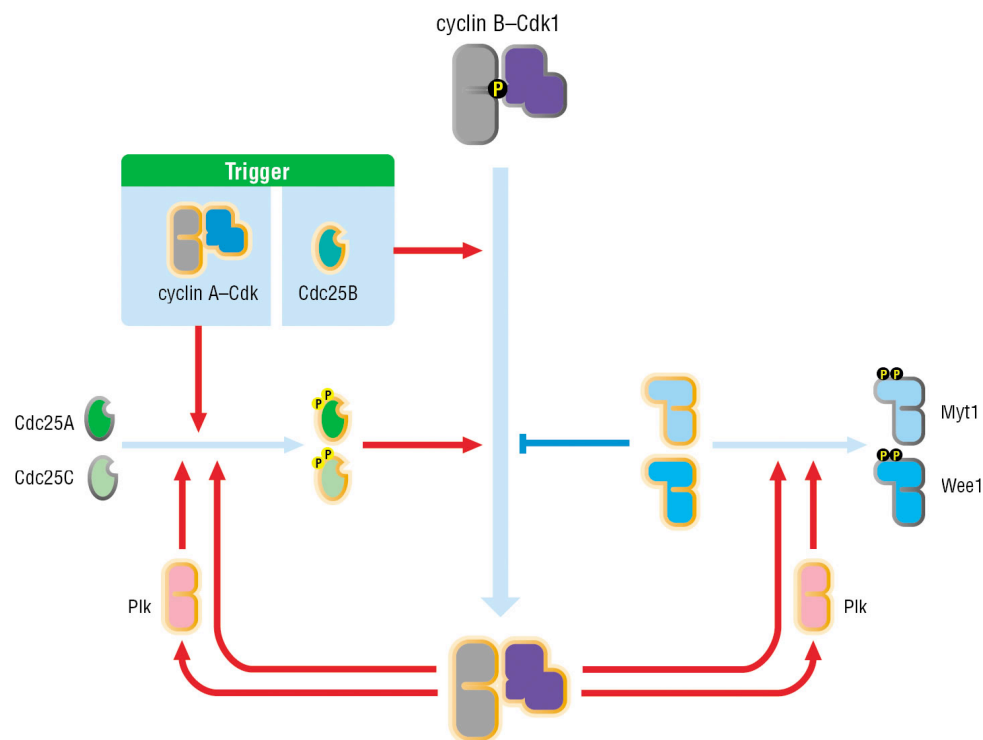


Figure 6- Multiple feedback loops regulate Cdk1 activity for mitotic entry. Cdk1 phosphorylates and activates Cdc25A and Cdc25C and inhibits Myt1 and Wee1. Cdk1 phosphorylation of Plk1 also stimulates Cdk1 activity. Cdc25B and cyclin A/Cdk complexes may trigger Cdk1 activation by promoting partial Cdk1 dephosphorylation in late G2. (Adapted from Morgan DO, *The Cell Cycle: Principles of Control*)

1.2.5. Mitosis

The duplicated genome is divided equally to the daughter cells during mitosis, to ensure that each cell receives a complete set of chromosomes. This is carried out under 5 different phases: prophase, prometaphase, metaphase, anaphase and telophase culminating with cytokinesis (see Fig. 7).

As cells complete replication, chromosomes undergo major structural changes resulting in their compaction, one of the prerequisites to the maintenance of fidelity in the process of chromosome segregation. This is mediated by DNA catenation with a specialized class of proteins called cohesins. The sister chromatids are held together by kinetochores at the centromeric region. As the cells enter prophase, chromosome condensation begins followed by centrosome separation in mid to late prophase. Chromosome condensation requires the protein complex called condensin and DNA decatenation by topoisomerase II (Ferrari, 2006). As cells progress to prometaphase, NEBD takes place allowing cytosolic microtubules to be in contact with nuclear chromatin. The kinetochores begin to be captured by the microtubules arising from the opposite poles of the forming spindle and this continues until all chromosomes have been attached. Even a single unattached kinetochore is capable of activating the spindle assembly checkpoint (Rieder et al., 1995) that reversibly arrests the cell cycle until all kinetochores are held by the microtubules. During metaphase, chromosomes congress to the centre of the cell to form the metaphase plate. This alignment ensures that upon division, each daughter cell will receive a complete set of chromosomes. Anaphase begins with the dissolution of cohesins mediated by the proteolytic activity of the anaphase promoting complex/cyclosome (APC/C) E3 ligase. APC^{Cdc20} targets degradation of securin, an inhibitor of the enzyme separase, allowing activation of separase and cleavage of the cohesin complexes (Castro et al., 2005). This is also accompanied by inactivation of Cdk1 through degradation of cyclin B1, further facilitating mitotic exit. Nuclear envelope reforms and spindles disassemble in telophase, which is followed by pinching off the two daughter cells, completing the process of mitosis.

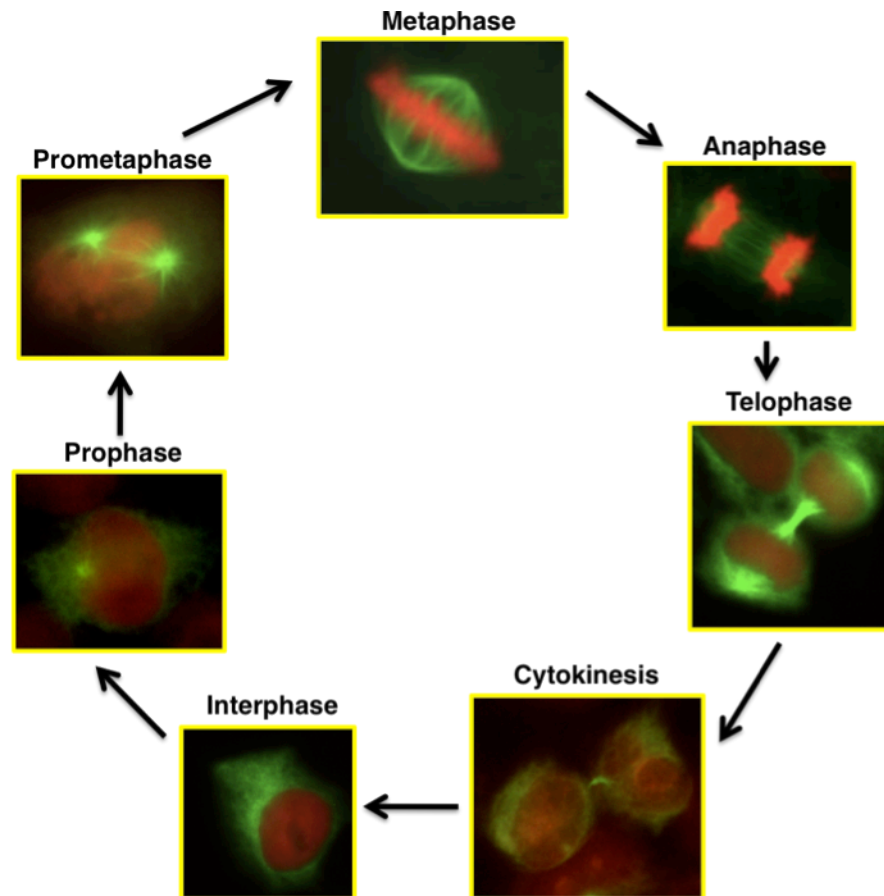


Figure 7- Mitotic events in HeLa cells. HeLa cell line stably expressing mGFP-tubulin and mCherry red-H2B plasmids was imaged using fluorescence microscopy to depict different mitotic phases. (See text for details).

1.3. CELL CYCLE REGULATION BY UBIQUITINATION AND PROTEIN DEGRADATION

1.3.1. Ubiquitin biology

Ubiquitin (Ub) is a small 76 amino-acids (8 kDa) protein that binds to the target proteins in a process called ubiquitination and regulates their function and degradation by the 26S proteasome pathway. This involves a 3-step reaction mediated via enzymes known as the E1 activating enzyme, E2-conjugating enzyme and the E3 ligase. In the first step, Ub is transferred to the E1-activating enzyme in an ATP-

dependent manner. Mechanistically, the E1 enzyme forms a thiol ester bond with the carboxyl group of G76 in Ub, the E2 enzyme carries the Ub transiently as a thiol ester and the E3 ligase transfers the Ub to the target protein by formation of an isopeptide bond between the carboxyl terminus of the Ub and the ϵ -amino group of the Lys in the target protein (Fig. 8) (Pickart, 2001). The Lys48 in Ub itself can be further conjugated by another Ub molecule, resulting in polyubiquitination (Huang and D'Andrea, 2006).

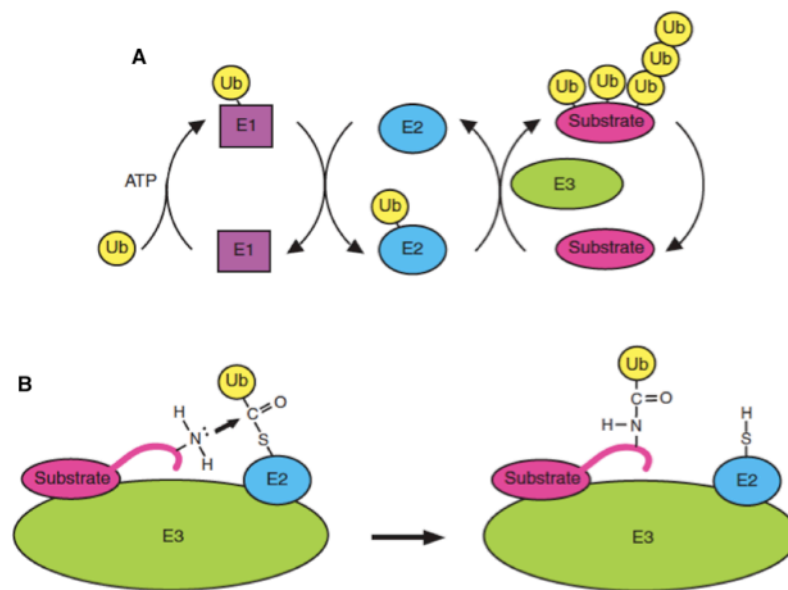


Figure 8- Mechanism of protein ubiquitination. A) Steps involving the three ubiquitin pathway enzymes: E1, E2 and E3. B) Detailed mechanism of the final step of ubiquitination. (See text for details). (Adapted from Matyskiela et al., 2009)

The extent of ubiquitination on the target protein decides whether or not it can be degraded via the 26S proteasome pathway. The Lys48-linked polyubiquitinated proteins, for example, are degraded while the Lys63 polyubiquitinated proteins play significant roles during the processes of signal transduction and DNA repair (Sun and Chen, 2004), (Huang and D'Andrea, 2006). Another modification is monoubiquitination that also does not target substrates for degradation. Monoubiquitinated proteins have been associated with functioning in diverse

processes such as DNA repair, gene silencing and vesicle sorting (Sun and Chen, 2004).

1.3.2. Ubiquitination and Cell cycle

Various proteins central to eukaryotic cell division are regulated by Ub-mediated degradation. The protein level of cyclins, Cdk inhibitors (CKI) and many other proteins oscillate throughout the cell cycle as a result of periodic and regulated proteolysis. Ub-ligases are the master regulators of this process as they serve as primary determinants of target/substrate specificity. Two large multisubunit E3 ligase complexes are involved in G1-S and metaphase-to-anaphase transition: the SCF (Skp1-Cul1-F-box) E3 ligase complex and the APC/C E3 ligase complex, respectively.

Structurally, the human SCF core complex consists of three invariable components: Skp1, Cul1 and the RING protein Rbx1 and a variable component called the F-box protein (Nakayama and Nakayama, 2005). The Rbx1 protein binds to the E2-conjugating enzyme and the target protein binds to SCF via the F-box protein; the latter therefore confers substrate specificity for the SCF complex (Fig. 9A). With a combination of more than 70 F-box proteins, the SCF core components and a number of E2 enzymes, multiple substrates can be targeted specifically (Nakayama and Nakayama, 2005). The APC complex also consists of a core component, the Apc2 and a RING subunit Apc11 (Fig. 9B) (Morgan DO, *The Cell Cycle: Principles of Control*). It is, however, much larger than the SCF complex with 11-13 Apc subunits present.

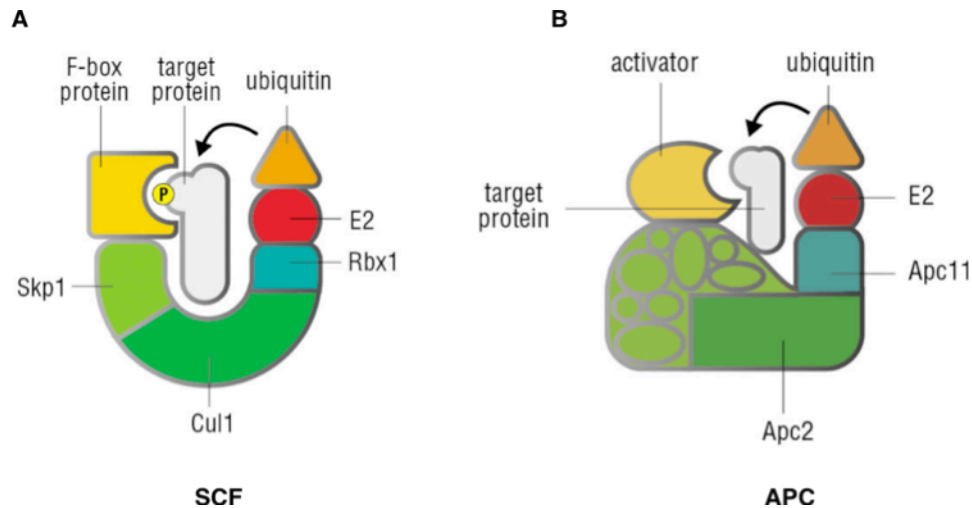


Figure 9-Multisubunit SCF and APC E3 ligase complexes. A) SCF complex consists of three core subunits: Skp1, Cul1 and Rbx1 and a variable component F-box protein. The Rbx1 interacts with the E2 enzyme and the F-box protein interacts with the phosphorylated substrate. B) APC complex also consists of an E2 interacting subunit Apc11 and binds to its substrates upon activation by the activator proteins. (Adapted from Morgan DO, *The Cell Cycle: Principles of Control*)

Degradation of proteins by these ligases is carried out essentially by two distinct activation strategies: activation of the target and activation of the E3 ligase itself (Reed, 2003). The former strategy is used by the SCF complex-mediated degradation. Substrates of SCF complex bind their F-box proteins only if they have been phosphorylated at a specific site or cluster of sites (Fig. 9A), for example, by Cdks (Morgan DO, *The Cell Cycle: Principles of Control*). Unlike SCF, the APC ligase itself must be activated in order to promote degradation. This is achieved by two activator subunits of the APC complex, known as Cdc20 (cell division cycle) and Cdh1 (Cdc20-dependent homolog). Once activated, these two complexes target a number of cell cycle proteins (Fig. 10) to ensure unidirectionality and faithful chromosome duplication and segregation.

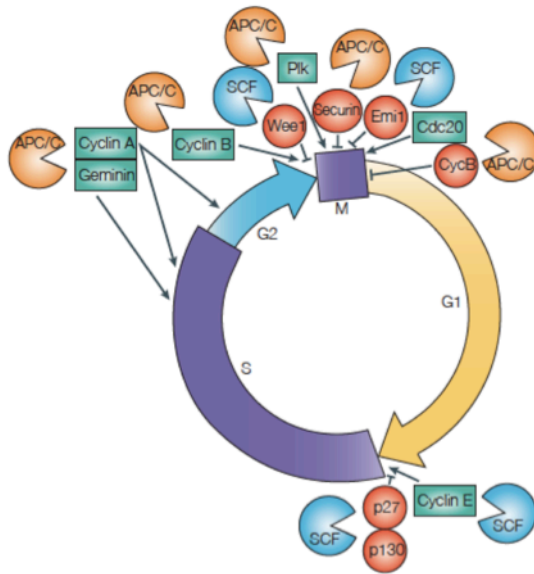


Figure 10- Important substrates of the SCF and APC E3 ligase complexes. These E3 ligases target various proteins at different phases of the cell cycle. (Adapted from Reed, 2003)

Here, I briefly describe the role of each of the two E3 ligases in regulating eukaryotic cell cycle events.

1.3.3. SCF E3 LIGASE

As mentioned above, the F-box proteins mediate substrate specificity for the SCF ligase complex. Several F-box proteins are known that degrade a huge number of proteins; of them, three main F-box proteins that are implicated in cell cycle control are Skp2, Fbw7 and β TrCP. A list of their substrates is shown in Fig. 11.

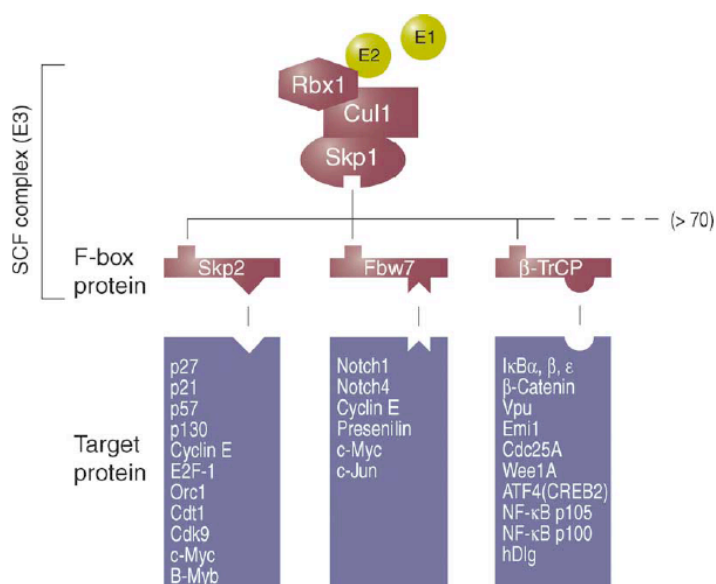


Figure 11- Substrates targeted for ubiquitination and degradation by different F-box proteins. (Adapted from Nakayama and Nakayama, 2005)

1.3.3.1. Functions of SCF E3 ligases

Skp2 is a growth promoter and oncoprotein that is thought to regulate the G1 and S phases by interacting with and promoting degradation of p27, p21 and p57 CKI (Nakayama and Nakayama, 2006). The Fbw7 protein is instead a tumor suppressor that targets several oncoproteins such as cyclin E and Myc for destruction (Nakayama and Nakayama, 2006). β TrCP is a more versatile protein as it targets proteins involved in many different pathways. Wnt signaling protein β -catenin is a known substrate of β TrCP (Nakayama and Nakayama, 2005). Among the proteins involved in cell cycle regulation, it targets Cdc25 phosphatases, Wee1 and Emi (early mitotic inhibitor), inhibitor of the APC/C complex (Nakayama and Nakayama, 2005). Degradation of Cdc25A via β TrCP occurs both during S-phase and in response to DNA damage. Phosphorylation of S82 in Cdc25A by Chk1/2 upon damage promotes its interaction with β TrCP for degradation (Busino et al., 2003). Recently, it is also shown to target Bora (protein required for AurA activation in G2) after its phosphorylation by Plk1 to facilitate mitotic entry and association of AurA with its mitotic partners (Seki et al., 2008a).

1.3.4. APC/C E3 LIGASE

As mentioned before, this is the major mitotic E3 ligase that is active during the metaphase-to-anaphase transition and throughout G1 to ensure degradation of cell cycle proteins and maintain order of the cell cycle events.

1.3.4.1. Regulation of APC/C

The activity of APC/C is regulated by binding of its activators Cdc20 and Cdh1 (Fig. 12). $\text{APC}^{\text{Cdc20}}$ is formed in early mitosis: the interaction of Cdc20 with APC is facilitated by phosphorylation of APC core components by cyclin B1/Cdk complex (Baker et al., 2007), while inhibitory phosphorylations at Cdh1 by the cyclin B/Cdk complex keep it from binding to APC at this time (Peters, 2002). APC/C activity is restricted until mitosis by Emi, binding of which allows accumulation of mitotic cyclins and in late G1 helps inactivation of APC^{Cdh1} to allow entry into S-phase

(Vodermaier, 2004). During G2 and early mitosis, Emi binds to Cdc20 preventing its association with the APC/C core complex. In prophase Plk1 phosphorylates and promotes the degradation of Emi via the SCF^{BT_{RC}P} E3 ligase, resulting in initiation of Cdc20 activation (Baker et al., 2007). Cdc20 is also regulated by the components of spindle assembly checkpoint (SAC) to prevent premature anaphase entry. SAC components contribute to this by both promoting phosphorylation and regulating the stability of Cdc20 (Fig. 12) (Ge et al., 2009).

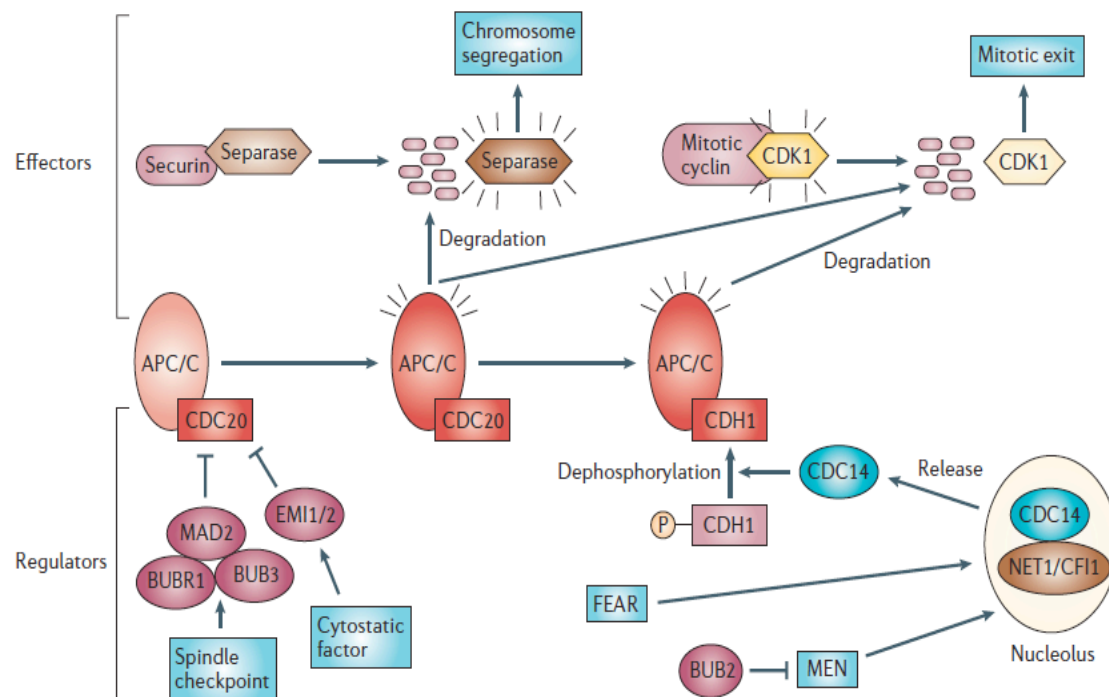


Figure 12- Regulation and function of APC/C complex during cell cycle. (See text for details). (Adapted from Nakayama and Nakayama, 2006)

The switch from APC^{Cdc20} to APC^{Cdh1} is essential to regulate late mitotic events and several pathways are engaged in manifesting this switch. Degradation of cyclin B initiated by APC^{Cdc20} results in decreased Cdk1 activity. This sets the stage for Cdh1 activation as Cdk1-dependent inhibitory phosphorylations in Cdh1 are reversed by

protein phosphatases. In budding yeast, this is accompanied by accumulation of Sic1, a CKI, that further dampens Cdk activity (Peters, 2002). The Cdc14 phosphatase dephosphorylates both Cdh1 and Sic1 to activate the former and stabilize the latter by preventing its recognition by the SCF E3 ligase complex (Peters, 2002). Once activated, APC^{Cdh1} also targets Cdc20 for destruction, resulting in a complete switch to APC^{Cdh1} proteolytic activities (Fig. 12).

1.3.4.2. Substrate recognition by APC/C

Targets of the APC/C complex present specific degrons that act as signals for recognition of substrates by the APC/C E3 ligase. These include mainly the D-box (destruction box) sequence RXXLXXXN and the KEN-box (Pfleger and Kirschner, 2000). The KEN-box sequence is preferentially recognized by APC^{Cdh1} (Pfleger and Kirschner, 2000). Although these sequences are required for degradation by the APC/C complex, many of its substrates possess unconventional sequences, indicating additional unidentified sequences are also involved in this process (Matyskiela et al., 2009).

1.3.4.3. Functions of APC/C

APC^{Cdc20} mainly targets two proteins, securin and mitotic cyclins to promote the onset of anaphase (Table II). Anaphase is marked by dissolution of the sister chromatids and their movement to the opposite spindle poles to initiate chromosome segregation. The sister chromatids are held together by a multiprotein complex called cohesin (Uhlmann et al., 1999). The enzyme separase that mediates cohesin cleavage is bound by its inhibitor securin until metaphase. In addition, phosphorylation of separase by the cyclin B/Cdk complex keeps it inactive in order to prevent premature sister chromatid separation (Baker et al., 2007). As soon as the SAC is inactivated and an active APC^{Cdc20} complex forms, it targets the destruction of securin to initiate anaphase. Cyclin B1 is also targeted for proteolysis by APC^{Cdc20} resulting in inactivation of Cdk1 (Baker et al., 2007) and reversal of Cdk1 mediated phosphorylations via protein phosphatase 1 (PP1) to facilitate mitotic exit (Wu et al., 2009).

Following the drop of Cdk1 activity, Cdh1 is activated and APC^{Cdh1} dependent proteolysis takes over. APC^{Cdh1} then targets several mitotic proteins for destruction (Table II), thereby triggering exit from mitosis. APC^{Cdh1} mediated proteolysis continues throughout G1, especially to maintain low Cdk activity, a necessary requirement for the loading of pre-replicative complexes at origins of replication (Nakayama and Nakayama, 2006). APC^{Cdh1} promotes degradation of geminin, an inhibitor of DNA replication factor Cdt1, allowing Cdt1 action on the origins (Nakayama and Nakayama, 2006).

Substrate	Start of degradation	APC/C activity involved
Cyclin A	prophase	APC/C ^{Cdc20} (early mitosis); APC/C ^{Cdh1} (G1)
Nek2A	prophase	APC/C ^{Cdc20}
Cyclin B	metaphase	APC/C ^{Cdc20} and APC/C ^{Cdh1}
Securin	metaphase	APC/C ^{Cdc20} and APC/C ^{Cdh1}
Xkid	metaphase	APC/C ^{Cdc20} and APC/C ^{Cdh1}
Prc1	metaphase	APC/C ^{Cdh1}
Kip1	metaphase	APC/C ^{Cdc20}
Cin8	metaphase	APC/C ^{Cdh1}
Geminin	metaphase	currently unknown
Tpx2	anaphase	APC/C ^{Cdh1}
Plk1	before mitotic exit	APC/C ^{Cdh1}
Aurora A	before mitotic exit	APC/C ^{Cdh1}
Cdc20	before mitotic exit	APC/C ^{Cdh1}
Aurora B	before mitotic exit	APC/C ^{Cdh1}
Anillin	before mitotic exit	APC/C ^{Cdh1}

Table II- List of mitotic substrates of APC/C during the cell cycle. (Adapted from Baker et al., 2007)

1.3.5. Interplay of SCF and APC/C during cell cycle

As both the E3 ligase complexes are active during the cell cycle (although at different times), an interesting question is whether or not they are coordinated at some point to regulate the cell cycle. Although SCF targets substrates from late G1 to early M and

APC/C from mid M to the next G1, there occurs a remarkable overlap of their functions to maintain the order of cell cycle events. This is manifested in the following way (Fig. 13): SCF ^{β TrCP} recognizes and degrades the APC/C inhibitor Emi1 after it has been phosphorylated by Plk1 in early mitosis. This event, in turn, results in activation of the APC/C mediated proteolysis. Later in G1, APC^{Cdh1} recognizes the D box of Skp2 and degrades it, thereby allowing accumulation of p27 and other CKIs for promoting DNA replication and S-phase progression. Increased expression of Skp2 at the G1-S boundary promotes p27 degradation and activates the S-phase cyclin/Cdks, which induce the dissociation of Cdh1 from the core APC/C subunits by the phosphorylation of Cdh1. As a result, APC/C activity declines at the G1-S boundary, allowing mitotic cyclins to accumulate gradually during the following S and G2 phases (Nakayama and Nakayama, 2006).

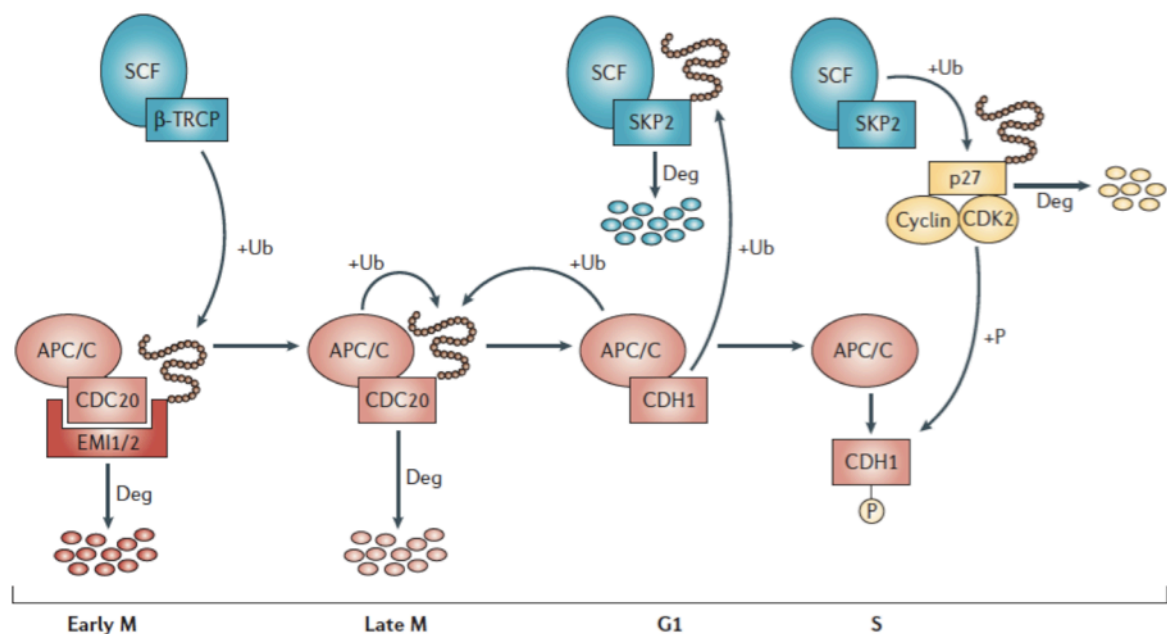


Figure 13- Coordination of cell cycle regulation by SCF and APC/C complexes.
(See text for details). (Adapted from Nakayama and Nakayama, 2006).

1.4. DNA DAMAGE/CELL CYCLE CHECKPOINTS

In order to ensure equal distribution of chromosomes to the daughter cells, different cell cycle checkpoints exist. By definition, a ‘*checkpoint*’ can be described as the guard that prevents the cell’s entry to next phase until the previous one is completed faithfully. A cell employs such a guard at the gate of every phase transition it makes. A failure of any one of these checkpoints results in a number of defects in essential processes like DNA replication, DNA damage repair, chromosome segregation and cytokinesis. The consequence of such failures (apart from apoptosis) is, in most cases, generation of chromosomal instability and aneuploidy, leading to cancer development.

Fig. 14 briefly summarizes all the checkpoints known till date and this chapter describes in detail the importance and functionality of each of these checkpoints.

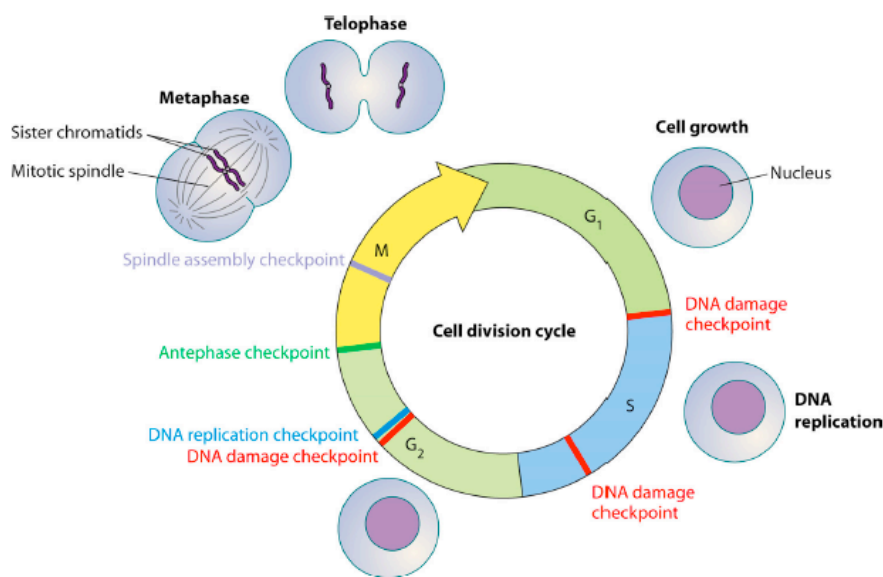


Figure 14- Schematic representation of different cell cycle checkpoints. Damage induced checkpoints are prevalent mainly at the G₁-S and the G₂-M phases (written in red). Other checkpoints are also present that monitor cell cycle progression in an unperturbed cell cycle and also in response to damage. (See text for details). (Adapted from Chin and Yeong, 2010)

The molecular components of the DNA damage-signaling pathway include the following categories of proteins:

- 1. Sensor proteins:** these proteins recognize the damage and include the Rad9-Hus1-Rad1 PCNA-like sliding clamp complex, Rad17-RFC clamp loading complex and the MRN (Mre11-Rad50-Nbs1) nuclease complex (Lukas et al., 2004). Also, Ku70/80 proteins act a sensor for NHEJ and recruit DNA-PK to the sites of damage.
- 2. Adaptors/Mediators:** these proteins associate with the sensors and signal transducers and provide specificity (Sancar et al., 2004). This class includes BRCA1, MDC1, 53BP1 and claspin (Lukas et al., 2004).
- 3. Signal transduction kinases:** these are of the PI3K-family of kinases and include ATM, ATR and DNA-PK (Lukas et al., 2004).
- 4. Effector kinases:** these are the ser/thr kinases including Chk1 and Chk2 (Lukas et al., 2004).
- 5. Effector proteins:** the final components include proteins that regulate cell growth, proliferation, cell cycle and apoptosis. These are the transcription factors p53 or E2F1, phosphatases of the Cdc25 family and protein kinases such as Cdks (Lukas et al., 2004), (Niida and Nakanishi, 2006).

All these proteins regulate the following cell cycle checkpoints to ensure faithful replication and chromosome segregation.

1.4.1. G1-S checkpoint

Damage induced in G1 cells prevents entry into S-phase by activation of protein kinases ATM/ATR that target two classes of proteins: the cell cycle phosphatases of Cdc25 family and the tumor suppressor transcription factor p53 (Lukas et al., 2004). Chk1/Chk2 effector kinases directly phosphorylate Cdc25A, promoting its degradation via the β TRCP ubiquitin ligase (Lukas et al., 2004), (Sancar et al., 2004). This blocks the activation of Cdk2 that associates with cyclin E and cyclin A to promote loading of Cdc45 onto the chromatin for recruitment of DNA polymerase α to initiate replication (Lukas et al., 2004). Consequently, a rapid p53-independent G1 arrest sets in.

The second pathway preventing S-phase entry is initiated much later, persists for a long time and requires p53. p53 is phosphorylated on S15 by ATM/ATR kinases and on multiple residues by Chk1/Chk2 kinases (Shiloh, 2003). This results in activation of p53 that promotes transcription of p21, a Cdk4 inhibitor. This, in turn, reduces the phosphorylation and suppression of pRB and thus S-phase promoting genes are no longer transcribed. Fig. 15 schematically describes the G1/S arrest.

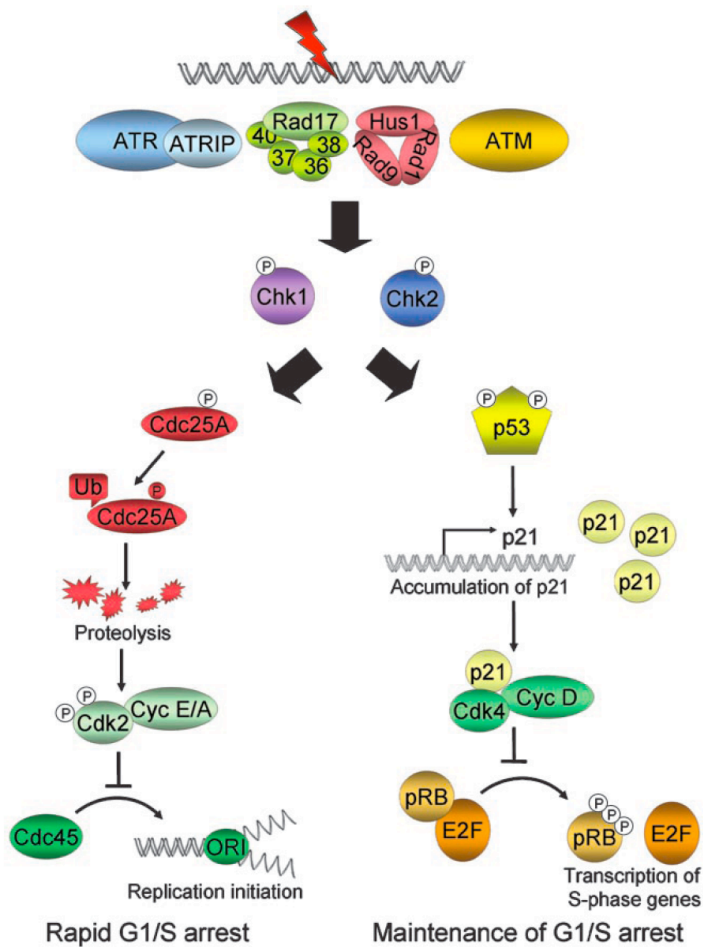


Figure 15- The G1/S checkpoint. DNA damage is sensed by ATM after double-strand breaks or by ATR, Rad17-RFC, and the 9-1-1 complex after UV-damage. ATM/ATR phosphorylates Rad17, Rad9, p53, and Chk1/Chk2 that in turn phosphorylates Cdc25A, causing its inactivation by nuclear exclusion and ubiquitin-mediated degradation. Phosphorylated and inactivated Cdk2 accumulates and cannot

phosphorylate Cdc45 to initiate replication. Maintenance of the G1/S arrest is achieved by p53 that induces p21^{WAF-1/Cip1} transcription. (Adapted from Sancar et al., 2004)

1.4.2. Intra S-phase checkpoint

Damage induced in cells going through S-phase or unrepaired damage from the G1 phase results in a delay in S-phase progression via the intra S-phase checkpoint. It is an extremely transient and p53-independent phenomenon. The execution of this checkpoint mainly occurs via the ATM-Chk1-Cdc25A-Cdk2-Cdc45 signaling cascade that is also involved in the G1 checkpoint mechanism (Lukas et al., 2004). Another pathway mediating the checkpoint function is via the ATM-dependent phosphorylation of SMC1 protein (Sancar et al., 2004). This, rather than being involved in arresting S-phase, primarily promotes recovery from damage with the help of proteins such as BRCA1 and Nbs1 (Sancar et al., 2004).

In response to UV-induced damage or stalled-replication forks, ATR-dependent pathways are activated. Inhibition of replication origin firing is one of the targets of ATR-dependent S-phase delay. This is executed by promoting downregulation of Cdc7-Dbf4 protein kinase activity that facilitates Cdc45 binding to DNA (Sancar et al., 2004). Thus, mechanisms controlling G1 to S phase transition and S-phase delay partially overlap with each other.

1.4.3. G2 checkpoint

Cells prevent entry into mitosis by prolonging the G2 phase in response to DNA damage. Although the G2 checkpoint activates several signaling pathways, it ultimately targets the cyclin B/Cdk1 complex (Fig. 16). In the presence of double-stranded breaks (DSBs), the ATM-Chk2 pathway is activated while the ATR-Chk1 pathway is preferentially involved in the response to DNA replication stress (Harper and Elledge, 2007). ATM/ATR-dependent phosphorylation of the downstream kinases Chk1 and Chk2 allows rapid transduction of the damage signal to effector proteins that control DNA repair, cell cycle progression and apoptosis (Kastan and Bartek, 2004). With regard to the cell cycle machinery, phosphorylation and activation of Wee1 and inhibition of Cdc25 phosphatases are the key events for inactivation of cyclin B/Cdk1, thus ensuring that cells do not go past G2 into prophase (Ferrari, 2006). Activation of Wee1 results in phosphorylation of two negative sites in

Cdk1 that block its catalytic activity. In parallel, Chk1/Chk2 driven phosphorylation of Cdc25C, the phosphatase responsible for dephosphorylation of the negative sites in Cdk1 ATP-binding domain, leads to 14-3-3-mediated nuclear export of the phosphatase (Sanchez et al., 1997), (Peng et al., 1997), thus blocking its function. It is known that in response to UV-induced damage, the p38 kinase mediates phosphorylation of Cdc25B, facilitating binding of 14-3-3 and blocking access of substrates to Cdc25B (Lukas et al., 2004). Collectively, this checkpoint ensures that Cdk1 is inactive and cells do not go past G2 into prophase.

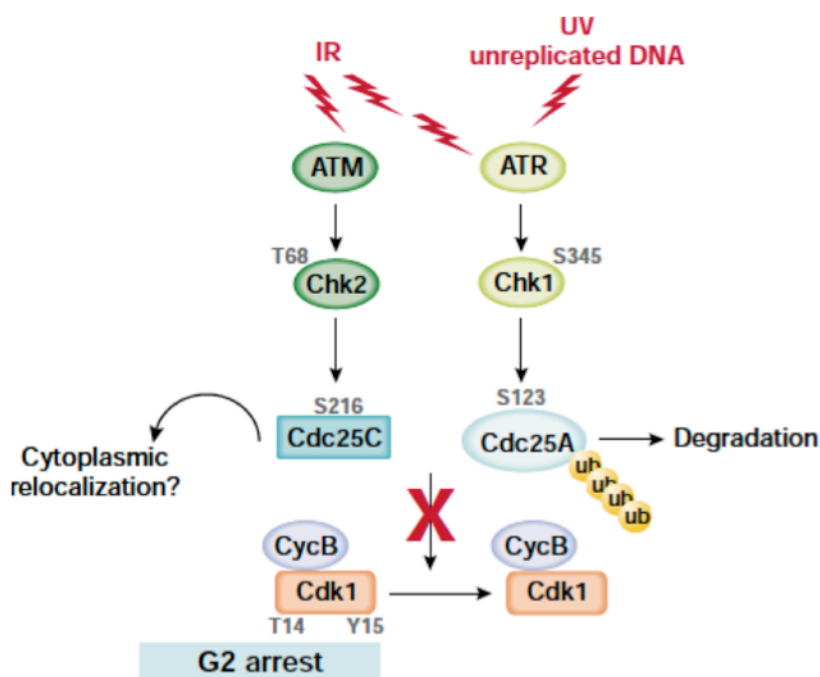


Figure 16- Schematic representation of G2 arrest. In response to DNA damage, ATM and ATR signaling pathways are activated that regulate the Cdc25 phosphatases to prevent activation of cyclin B-Cdk1 complex and thereby maintain a G2 arrest. (Adapted from Donzelli and Draetta,

2003)

1.4.4. Decatenation checkpoint

Decatenation or disentanglement of duplicated chromosomes occurs after replication to ensure that each daughter cell gets a copy of the genome accurately. This is absolutely essential for an error-free cell division. The process of decatenation is mediated by a class of enzymes called topoisomerase II (topo II), that, in a two-step reaction allow the two DNA duplexes to be separated from each other (Wang, 1996). A G2 phase decatenation checkpoint functions to prevent entry into mitosis until the

DNA has been decatenated completely (Downes et al., 1994). This checkpoint, however, is very distinct from the DNA damage checkpoint that prevents entry into mitosis in response to DNA DSBs. Identification of this as an independent checkpoint owes to the existence of two different classes of topo II inhibitors that have different effects on the enzyme. Inhibitors like etoposide (VP-16) and adriamycin produce massive DSBs in DNA that activate the G2 checkpoint. Instead, catalytic inhibitors like ICRF-193 and ICRF-187, when given in G2, do not produce DSBs but still cause a mitotic delay and thus led to the identification of a checkpoint mechanism different from the damage checkpoint (Downes et al., 1994). The catalytic inhibitors hold the enzyme in a closed clamp form tethered to DNA, in which the enzyme is unable to cleave DNA strands due to inhibition of its ATPase activity. This is different from the action of the classic topo II inhibitors that stabilize the topo II DNA-cleavable complex, resulting in the production of massive DSBs and hence elicit a damage response. As mentioned above, the signals of DNA damage reach the target proteins in an ATM/ATR-dependent manner. The ATR kinase plays a major role upon administration of topo II catalytic inhibitors, as cells defective in ATR do not show a mitotic delay even in the presence of ICRF-193 (Deming et al., 2001). Nonetheless, the involvement of ATM cannot be completely ruled out as caffeine, an inhibitor of both ATR and ATM kinases, fails to cause a mitotic delay in the presence of ICRF-193 (Downes et al., 1994). Furthermore, ATM activation in response to ICRF-193 occurs in human lung cancer cell lines (Nakagawa et al., 2004). The mechanism of an ATR-dependent signaling, however, is better defined and is known to occur through inhibition of Plk1, which phosphorylates cyclin B1 to promote its nuclear localization allowing mitotic entry (Deming et al., 2001). Therefore, abrogating ATR function would not cause a mitotic delay even if the decatenation function of topo II is inhibited.

In addition to the role of ATR, the RecQ helicase Werner (WRN) is also involved in this checkpoint mechanism. Cells lacking functional WRN show no mitotic delay in the presence of ICRF-187 and the checkpoint can be restored following ectopic expression of the helicase. Furthermore, in WRN deficient cells, there occurs more chromosomal damage and apoptosis as compared to the WT cells (Franchitto et al., 2003). BRCA1 is also an essential component of this checkpoint, as BRCA1-mutant

cells do not have a completely activated decatenation checkpoint (Deming et al., 2001). Still, more studies are needed to unambiguously define the roles of these proteins as contradicting results are available that deny the contribution of these additional proteins in decatenation checkpoint functioning (Nakagawa et al., 2004).

1.4.5. Antephase checkpoint

The term ‘antephase’ was coined by Bullough and Johnson (1951) and by definition described as a checkpoint that exists between late G2 and early prophase when the first signs of chromosome condensation start to appear (Matsusaka and Pines, 2004). A hallmark of this checkpoint is that cells exposed to various stresses in early prophase, specifically before NEBD begins, revert to an interphase-like state and do not progress into mitosis. Early prophase cells exposed to DNA damage (Carlson, 1969a), (Carlson, 1969b) or microtubule poisons (Rieder et al., 1995) showed reversible chromosome decondensation and reversion to G2, owing to the presence of the antephase checkpoint. This checkpoint, however, is essentially different from the classical G2 checkpoint, as it does not employ the PI3K-like kinases, ATM and ATR and is not activated in response to DNA damaging agents. The execution of this checkpoint, instead, depends on two proteins: CHFR (checkpoint with FHA and RING finger domains) E3 ligase and the p38 stress kinase (Matsusaka and Pines, 2004) and therefore is sometimes referred as the CHFR-mediated prophase checkpoint (Privette and Petty, 2008). The E3 ligase function of CHFR but not the proteasome is required for this checkpoint. Cells that lack a functional CHFR, e.g. HeLa and U2OS, progress into mitosis even in the presence of microtubule poisons (Scolnick and Halazonetis, 2000). CHFR actively participates in delaying mitotic entry by retaining the cyclin B1/Cdk1 complex in the cytoplasm in response to microtubule poisons (Privette and Petty, 2008) and interfering with chromosome condensation and NEBD (Scolnick and Halazonetis, 2000). Another way by which CHFR acts is by prolonging the inhibitory phosphorylation of Y15 in Cdk1 to prevent mitotic entry (Kang et al., 2002).

Inhibition of p38 kinase function also abrogates the antephase checkpoint (Matsusaka and Pines, 2004) and CHFR is known to act upstream of p38 kinase (Privette and

Petty, 2008). The p38 kinase-dependent antephase checkpoint is activated in response to various stresses like, UV radiation (Bulavin et al., 2001), osmolality changes (Dmitrieva et al., 2002) and changes in chromatin structure (Mikhailov et al., 2004). All these data suggest a very important role of CHFR and its downstream targets in preventing mitotic defects in response to a number of stresses before the cells reach a point of ‘no-return’ in mitosis (Fig. 17).

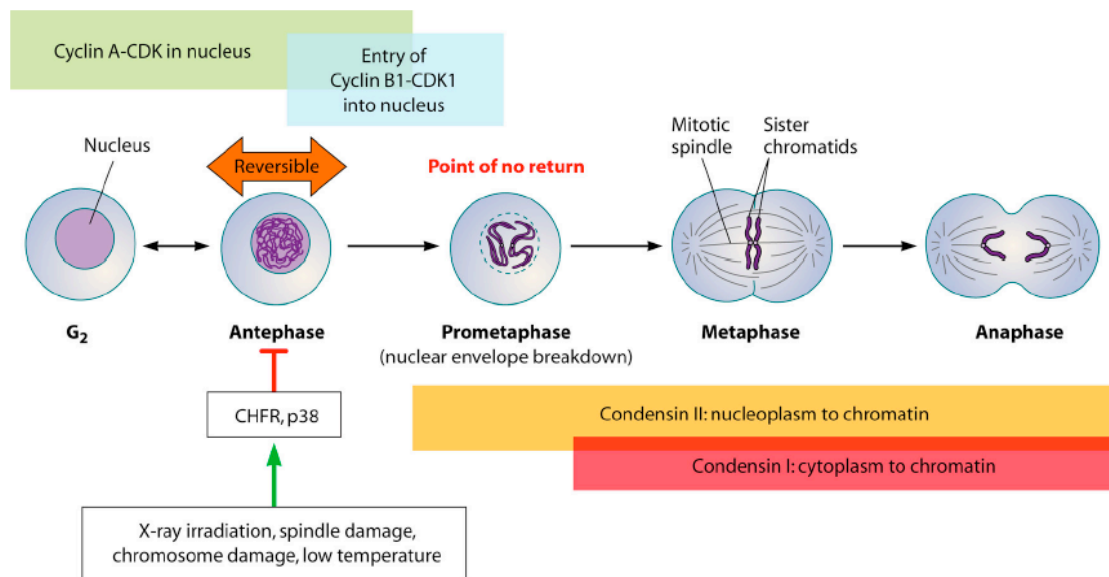


Figure 17- The antephase checkpoint. (See text for details). (Adapted from Chin and Yeong, 2010)

1.4.6. Mitotic checkpoint

1.4.6.1. DNA damage during mitosis

Unlike the well defined G₁ and G₂ phase checkpoints, there is no formal mitotic checkpoint that monitors DNA damage occurring in mitosis. If damaged in mitosis, cells mount a different response depending on the stage of mitotic progression. Early prophase cells can revert back to G₂ (Chow et al., 2003) and late prophase cells can arrest in prometaphase (Choi and Lee, 2008) or metaphase if the damage is severe

(Mikhailov et al., 2002) owing to the presence of spindle assembly checkpoint (see below). Damage in prometaphase and metaphase does not impede cell cycle progression but gives rise to chromosome segregation defects (Results Part 1 and 2 of this thesis). What is the ultimate fate of mitotic cells upon DNA damage? This long-standing question in the field has been recently answered by the group of S. Jackson who showed that mitotic damage, although sensed by the cells, is repaired only in the subsequent G1 due to inaccessibility of the damaged structures in highly condensed mitotic chromosomes to the signaling and repair proteins (Giunta et al., 2010). The only well-defined mitotic checkpoint is the spindle assembly checkpoint, discussed in detail in the next section.

1.4.6.2. Spindle Assembly Checkpoint (SAC)

As cells progress through mitosis, SAC ensures the fidelity of chromosome segregation. SAC is activated when spindle assembly is interfered with. It acts as both an intrinsic checkpoint to monitor stable kinetochore (KT)-microtubule (MT) interactions and is also activated in response to extrinsic stresses such as presence of DNA damage and microtubule poisons. The primary reason of activation of SAC is lack of tension between KT-MT that arises due to improper connections. The main function of SAC is to arrest the cell cycle in prometaphase (when KT-MT interactions begin to ensure bi-orientation in metaphase) until all chromosomes are attached to the MTs arising from opposite poles. The main players of SAC include the MAD (mitotic arrest deficient, MAD1, 2 and 3) and BUB (budding uninhibited by benzimidazole, BUB1) family of proteins initially identified in yeast genetic screens as mutations that failed to arrest cell cycle in the presence of MT poisons (Hoyt et al., 1991), (Li and Murray, 1991). MAD2, MAD3 (BUBR1) and BUB3 along with Cdc20 form a mitotic checkpoint complex (MCC) that targets Cdc20 to prevent polyubiquitination and degradation of securin and cyclin B1 (Musacchio and Salmon, 2007). At the end of metaphase, Cdc20 mediated degradation of securin activates separase that allows sister chromatids to be pulled apart to the poles for equal segregation (Musacchio and Salmon, 2007). Proteolytic destruction of cyclin B1 allows inactivation of Cdk1 thereby promoting mitotic exit (Musacchio and Salmon, 2007). Recently, Cdc20 destruction was shown to be regulated by the MAD2 protein (Ge et al., 2009). MAD2

binds to Cdc20 and promotes its APC/C mediated degradation, allowing activation and maintenance of SAC in response to spindle poisons.

Apart from these components, the SAC also employs the mitotic kinase Aurora B as one of its effector proteins. Syntelic (i.e., both sister kinetochores are attached to MTs emerging from the same pole) and merotelic (i.e., one kinetochore is bound by MTs from both the poles) attachments are corrected by Aurora B and require proteins like Ndc80 (Nezi and Musacchio, 2009). In an unperturbed mitosis, conditions favoring merotelic arise frequently and are not sensed by SAC. They are therefore solely taken care of by Aurora B dependent correction mechanisms.

1.4.6.2.1. Role of CHFR as a checkpoint

CHFR is a known tumor suppressor protein with functions in mitotic checkpoint and as an E3 ligase. In addition to its role in mitotic stress prophase checkpoint (section 1.4.5), it is also activated as a part of the SAC. Downregulation of CHFR results in several defects in chromosome segregation and cytokinesis and interferes with proper metaphase kinetochore localization of SAC proteins Mad2 and BUBR1 (Privette and Petty, 2008). The CHFR protein is also suspected to be a target of APC^{Cdh1} due to the presence of a KEN motif (Privette and Petty, 2008). Thus, it is believed to have many mitotic checkpoint functions and thought to be regulated in a similar way like other mitotic proteins.

1.5. AURORA KINASES

Aurora kinases are a family of highly conserved ser/thr protein kinases that are involved in different processes of cell cycle progression. They are required for events such as mitotic entry, centrosome separation, assembly of bipolar spindle apparatus, chromosome compaction and segregation, spindle assembly checkpoint and cytokinesis.

The *Aurora* gene was first identified from *Drosophila melanogaster* in a screen for mutants that were defective in spindle-pole behavior (Glover et al., 1995) and from

S.cerevisiae, Ipl1 (increase in ploidy 1), in a screen for mutants defective in chromosome segregation (Chan and Botstein, 1993). Two *Aurora* genes are present in *Drosophila*, *C.elegans* and *Xenopus* and one in each fission (*S.pombe*) and budding yeast (*S.cerevisiae*) (Fu et al., 2007). The Aurora family in humans includes three members, namely A, B and C. The human Aurora kinases are highly conserved in their catalytic domains, with 67-76% identity, but have a varying N-terminal region (Bischoff and Plowman, 1999). The activation loop present in the catalytic domain contains a highly conserved thr (T) residue that is responsible for activation of the kinase (Fig. 18).

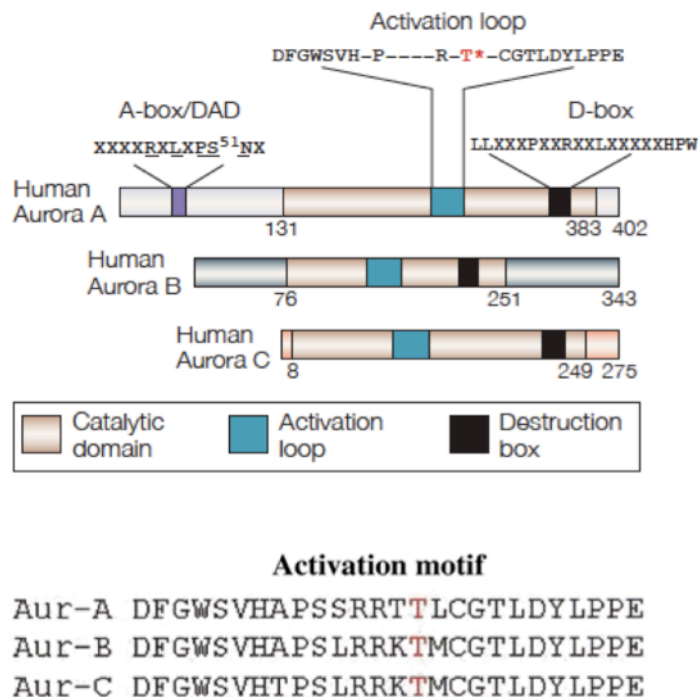


Figure 18- Structure of Aurora kinases. Upper panel: the position of various regions in Aurora kinases is shown. Asterisk marked T represents the thr residue whose phosphorylation regulates the activity of Aurora kinases. (Adapted from Carmena M. and Earnshaw C., 2003). Lower panel: Sequence of

the activation motif is shown highlighting the presence of the highly conserved thr residue. (Adapted from Katayama et al., 2003)

Despite their sequence similarity, the three proteins have different sub-cellular localization and functions. This is attributed to the different set of proteins they interact with throughout mitosis (Table III).

	Substrate	Cellular localization	Functions
AurA	PP1, p53, Cdh1, TPX2, Ajuba, RasGAP, BRCA1, TACC3, HURP, CENP-E	Centrosome, mitotic spindle	Centrosome maturation and separation, spindle assembly, chromosome compaction and cytokinesis
AurB	Histone H3, INCENP, CENP-A, vimentin, MCAK, survivin	Centrosome, central spindle, chromosome arms	Chromosome alignment and segregation, cytokinesis, MT dynamics, SAC
AurC	AurB, INCENP	Central spindles, chromosome arms	Spermatogenesis, SAC, cytokinesis

Table III- Aurora kinases interact with different proteins, have different sub-cellular localizations and perform different functions during mitosis. (Adapted from Bolanos-Garcia, 2005)

Apart from the conserved T-loop residue, the Aurora kinases also possess specific motifs that control their degradation once they are no more required. Aurora proteins are degraded by the mitosis specific APC/C E3 ligase in a proteasome dependent manner. APC/C mediated destruction of proteins requires the presence of conserved motifs, such as the KEN-motif, the D (destruction)-box and a GxENbox (Castro et al., 2005). Aurora A and B possess both the D-box and the D-box activating box (DAD/A-box), while Aurora C lacks the complete A-box sequence (Fig. 18). The

three kinases are regulated throughout the cell cycle by reversible phosphorylations and protein-protein interactions, although, least is known about Aurora C.

In this section, I briefly review Aurora B and Aurora C and provide a detailed review of function and regulation of Aurora A.

1.5.1. Aurora C (AurC)

AurC is the third member of the human Aurora kinase family identified by library screening of kinases expressed in mouse sperm and eggs (Tseng et al., 1998) and homology search with AurB (Kimura et al., 1999). AurC shares 83% homology with AurB and only 71% with AurA (Katayama et al., 2003). Like other members of the family, expression of AurC at the mRNA and protein level peaks during the G2-M transition (Katayama et al., 2003). The region to which *AurC* gene maps is translocated and deleted in many human cancers and also AurC is overexpressed in many human cancer cell lines (Bernard et al., 1998), (Kimura et al., 1999). AurC is highly expressed in mammalian testis (Tseng et al., 1998) (Bernard et al., 1998) (Kimura et al., 1999) and is associated with the function of spermatogenesis (Kimmins et al., 2007). Human AurC localizes to the centrosomes during mitosis, suggesting a role in centrosome regulation (Kimura et al., 1999). It has recently been shown to localize to centromeres and to the spindle midzone at anaphase in mouse oocytes. Deletion of AurC resulted in production of large polyploid oocytes, indicating a function in cytokinesis (Yang et al., 2010). During mitosis, human AurC is capable of complementing the functions of AurB, as it is also a known member of the chromosome passenger complex (CPC) and has been shown to interact with INCENP an AurB activator (Li et al., 2004). AurC directly binds to survivin and is required for cytokinesis (Yan et al., 2005) Furthermore, AurC is regulated by an inhibitory phosphorylation at T171 by PKA (Chen and Tang, 2002). Thus, AurC likely performs many functions that were initially associated with AurB. More work, however, is required to understand the regulation of AurC during mitosis and meiosis.

1.5.2. Aurora B (AurB)

Human AurB was identified in a PCR reaction screen for kinases overexpressed in tumors (Bischoff et al., 1998). Like AurA, it is also cell cycle regulated with expression and activity peaking at mitosis (Terada et al., 1998), (Bischoff et al., 1998). AurB is a component of the CPC complex along with INCENP, survivin and borealin that regulate mitosis in several ways (Carmena and Earnshaw, 2003). AurB is regulated by phosphorylation and interaction with other proteins. Binding of INCENP to AurB increases its kinase activity and is additionally responsible for proper localization of AurB to centromeres during mitosis (Katayama et al., 2003). The *C.elegans* Tousled-like kinase (TLK1) is both a substrate and activator of AurB (Han et al., 2005). Moreover, the checkpoint kinase Chk1 phosphorylates AurB and increases its activity at least *in vitro*, contributing to its activation in response to taxol (Zachos et al., 2007). AurB was shown to be highly active in the presence of a phosphatase inhibitor, okadaic acid (Sugiyama et al., 2002), indicating the requirement for phosphorylation in its activation. AurB interacts with phosphatases such as PP1 and PP2A that dephosphorylate it at the end of mitosis (Sugiyama et al., 2002), (Sun et al., 2008). The microtubule end-binding protein (EB1) shields the T-loop of AurB from dephosphorylation by PP2A (Sun et al., 2008).

AurB is involved in several different mitotic events, such as chromosome bi-orientation, chromosome condensation and segregation, SAC and cytokinesis (Carmena and Earnshaw, 2003). Bi-orientation is important to ensure enough tension is generated from the captured kinetochores. During an unperturbed mitosis, incorrect attachments such as monotelic and syntelic attachments occur naturally. AurB detects such improper attachments by phosphorylating and regulating substrates like Hec1/Ndc80 and MCAK (mitotic centromere-associated kinesin) (Carmena et al., 2009). AurB regulates KT-MT interactions by phosphorylating and recruiting kinetochore components such as CENP-E (Murata-Hori and Wang, 2002), (Kim et al., 2010). AurB is also an essential component of SAC and maintains the checkpoint under condition of low tension arising due to incorrect KT-MT attachments (Carmena and Earnshaw, 2003). It is negatively regulated by BubR1, one of the SAC components, to facilitate stable KT-MT interaction (Lampson and Kapoor, 2005).

AurB facilitates condensation of mitotic chromosomes by phosphorylating histone H3 at S10 (Gurley et al., 1978) and S28 (Goto et al., 2002). AurB also controls sister-chromatid cohesion by regulating the localization of the shugoshin protein (Carmena et al., 2009). Recently, AurB was shown to regulate the recruitment of separase to mitotic chromosomes (Yuan et al., 2009), an enzyme that is responsible for dissolution of the cohesin complexes at the end of metaphase.

AurB is also required for late mitotic events. It re-localizes from the centromeres to the spindle midzone in anaphase and finally to the midbody of the dividing cell. AurB regulates the contraction of the acto-myosin ring by phosphorylating the centralspindilin complex (Carmena et al., 2009). The kinase is also required for regulating abscission timing, as active AurB persists at the chromatin bridge formed between the daughter nuclei (Steigemann et al., 2009). AurB phosphorylates vimentin at S72 and this is required for cytokinesis (Goto et al., 2003). AurB also regulates proteins like MgcRacGap, MKLP-1 and condensin I in order to facilitate events of chromosome resolution and cytokinesis (Kitzen et al., 2010). Thus, AurB functions in early to late mitotic events by interacting with different proteins (Fig. 19), distinct from those of AurA (see below).

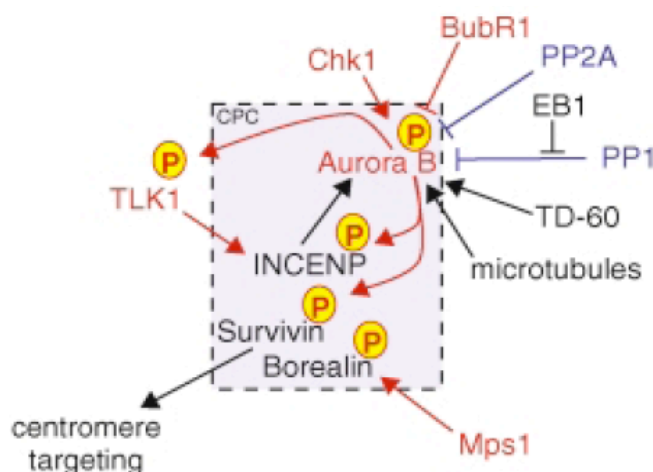


Figure 19- AurB regulators during mitosis. Kinases are shown in red, phosphatases in blue and phosphorylation events are depicted with arrows. (See text for details). (Adapted from Carmena et al., 2009)

1.5.3. Aurora A (AurA)

AurA is the first member of the human Aurora kinase family identified from *Drosophila* mutants that were defective in spindle-pole behavior (Glover et al., 1995). The gene-encoding AurA is located on chromosome 20 and this region is often amplified in several human tumors. The gene codes for a 403 amino acid protein of molecular weight of 46 kDa. AurA differs significantly from AurB and C with respect to its localization and function during mitosis. The protein is activated by phosphorylation of the T288 residue situated in the catalytic domain. AurA carries out important functions during mitosis by interacting with a number of different proteins (Fig. 20). Its downregulation results in delayed mitotic entry, monoploar spindles and defects in chromosome condensation (See Results Part 3).

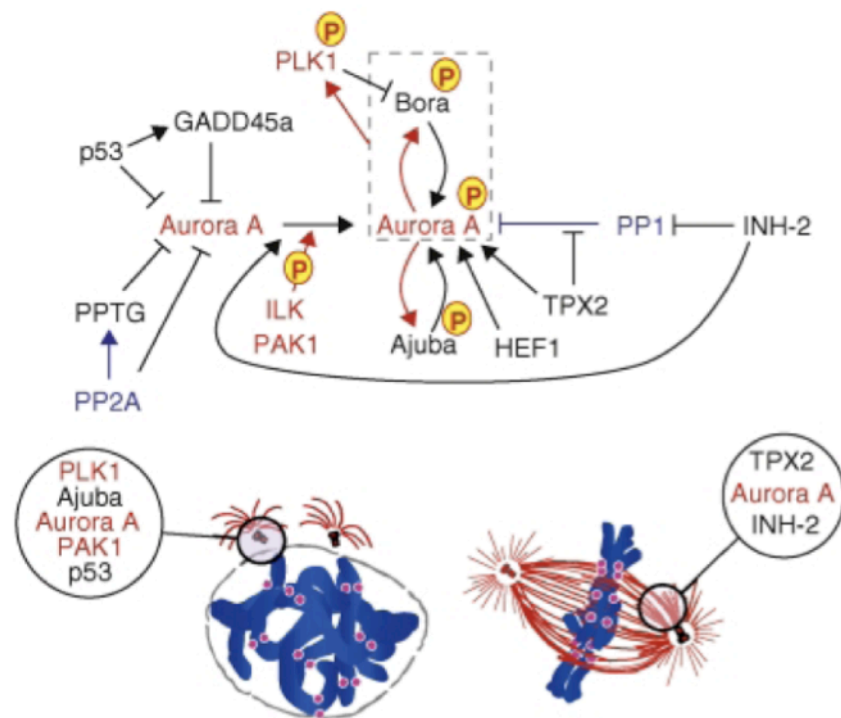


Figure 20- AurA regulators during G2 and mitosis. Kinases are shown in red, phosphatases in blue and phosphorylation events are depicted with arrows. (See text for details). (Adapted from Carmena et al., 2009)

In this section, I review the mechanisms of regulation of AurA and its functions in details.

1.6. FUNCTIONS OF AURORA A

AurA is involved in several different events required for both entering mitosis and mitotic progression. Here, I briefly describe the main roles of AurA in cell cycle progression.

1.6.1. Mitotic entry

AurA is required for the G2 to M-phase transition and multiple mechanisms of this have been suggested. Cdk1 is the *bona fide* G2-M kinase whose activation drives mitotic entry. Cdk1 is activated by the concerted actions of Cdc25 phosphatases (Murray, 2004) and mitotic kinases such as AurA and Plk1 (Lenart et al., 2007), (Hansen et al., 2004). AurA is shown to facilitate mitotic entry either by directly regulating the activity of Cdk1 (Krystyniak et al., 2006), (Liu and Ruderman, 2006) or indirectly through phosphorylation of Cdc25B (Dutertre et al., 2004), placing it upstream of Cdk1. Recently, however, Cdk1 was shown to mediate activation of AurA at the G2 to M-phase transition (Van Horn et al., 2010). A more defined mechanism for AurA regulated mitotic entry exists that involves its G2-interactor Bora (Seki et al., 2008b) Bora was shown to interact with and activate AurA in G2, resulting in the phosphorylation of T210 in Plk1 at the G2-M transition. This, in turn, triggers the Plk1-Cdc25-Cdk1 positive feedback loop leading to activation of Cdk1 and mitotic entry (Seki et al., 2008b).

1.6.2. Mitotic progression

AurA protein level and kinase activity increase from G2 until end of mitosis (Bischoff et al., 1998), (Krystyniak et al., 2006) (Results Part 1 of this thesis) and is associated with important events governing mitotic progression. AurA is associated with the centrosomes and spindle MTs throughout mitosis (Fig. 21). It plays an important role in **centrosome maturation** by phosphorylating TACC (transforming acidic coiled-

coil), which is then recruited to the centrosomes and interacts with microtubule-associated protein (MAP) Msps/ch-TOG that promotes the growth of MTs (Marumoto et al., 2005), (Kinoshita et al., 2005) AurA also helps in **centrosome separation** by phosphorylation of Eg5, a kinesin-like motor protein (Giet et al., 1999). Interaction of AurA with TPX2 results in its recruitment to the spindle MTs where it facilitates **assembly of bipolar spindles**. AurA is also associated with **chromosome alignment** at the metaphase plate. AurA phosphorylates CENP-A, a kinetochore-specific H3 variant and promotes proper KT-MT attachment required for chromosome alignment and segregation (Marumoto et al., 2005), (Kunitoku et al., 2003). Recently, AurA has also been shown to phosphorylate CENP-E, a kinetochore motor protein (Kim et al., 2010). This releases PP1 from CENP-E and allows congression of polar chromosomes towards the centre of the cell, thereby ensuring proper chromosome alignment at the metaphase plate. AurA also plays a role in regulating **late mitotic events** as depletion of AurA from metaphase cells results in abnormal cytokinesis and generation of multinucleated cells (Marumoto et al., 2003). Thus, by phosphorylating different proteins, AurA regulates their localization and function during mitosis.

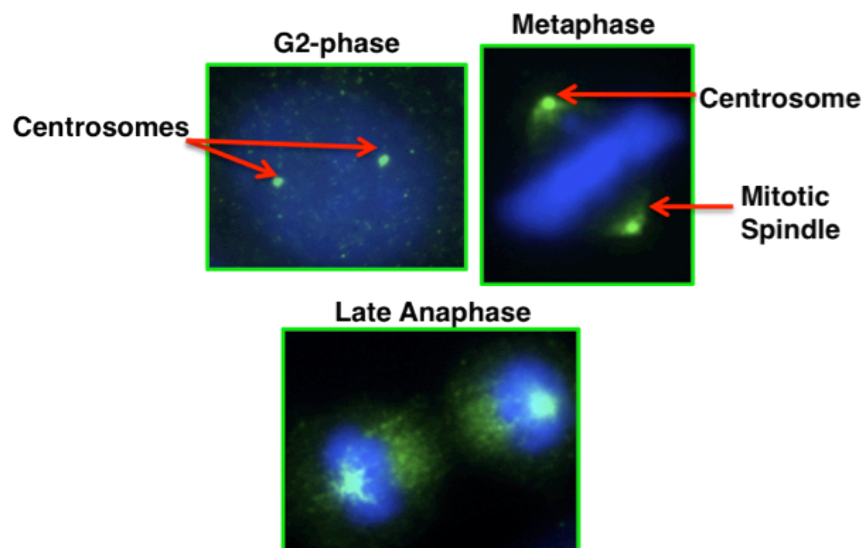


Figure 21 – Localization of AurA. AurA is localized to the centrosomes in G2 and to the spindle microtubules during early and late mitotic events. HeLa cells were stained with DAPI and AurA specific antibody and imaged using fluorescence microscopy.

1.6.3. AurA and cancer

AurA is considered a potential oncogene as it is found amplified in numerous human tumors (Bischoff et al., 1998), (Zhou et al., 1998), (Tanner et al., 2000). Both gene amplification and overexpression of AurA have been linked to cellular transformation. Overexpression of AurA in mouse NIH-3T3 gives rise to tumors when injected in nude mice (Bischoff et al., 1998), (Zhou et al., 1998). Overexpression of AurA has been associated with resistance to taxol (Anand et al., 2003), cisplatin and UV (Wang et al., 2006). How AurA promotes tumorigenesis is, however, not very clear. One of the suggested mechanisms is disruption of cell cycle checkpoints. Both AurA gene amplification and overexpression are shown to override the SAC triggered by nocodazole (Jiang et al., 2003) and taxol (Anand et al., 2003). This has shown to be independent of kinase activity (Jiang et al., 2003) and to result in an abnormal cytokinesis leading to centrosome amplification, multinucleation and ultimately to aneuploidy. Another mechanism contributing to AurA mediated aneuploidy is via tetraploidization due to deregulated cytokinesis and centrosome amplification (Marumoto et al., 2005), (Meraldi et al., 2002). This response is amplified in cells with dysfunctional p53 where cells fail to arrest in the subsequent G1, entering another round of replication (Meraldi et al., 2002). Also direct regulation of p53 stability and function by AurA favors aneuploidy in AurA overexpressed environment.

Fig. 22 summarizes the mechanisms of AurA-dependent aneuploidy.

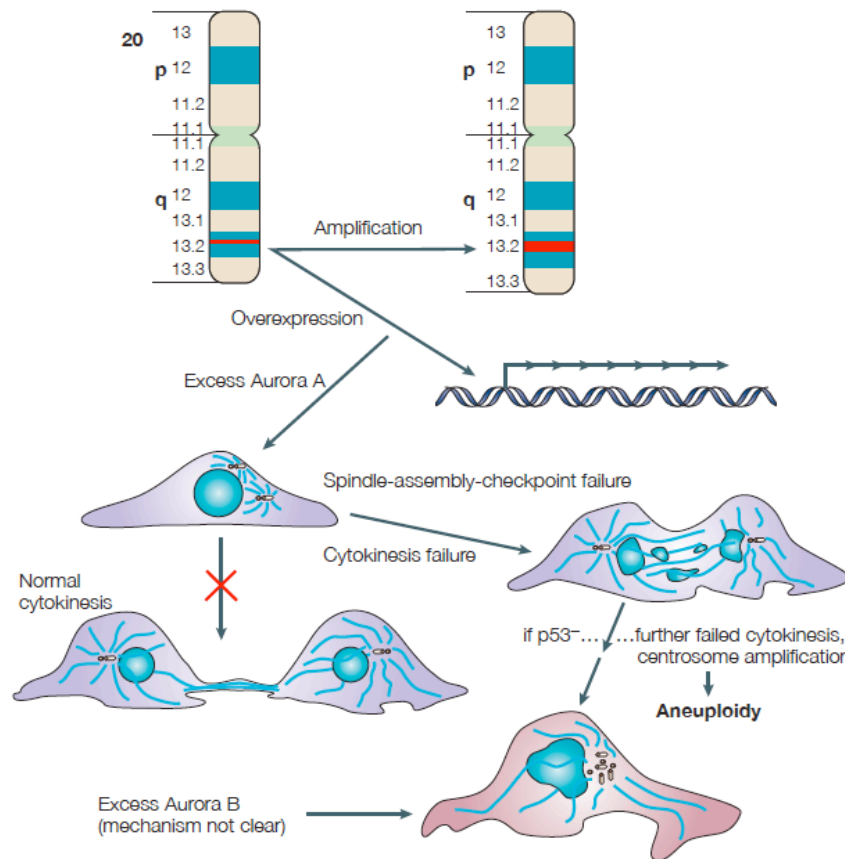


Figure 22- Overexpression of AurA leads to aneuploidy. Overexpression of AurA, either as a consequence of gene amplification or by other means, disrupts mitotic progression, apparently by blocking the ability of chromosomes to achieve a normal orientation on the spindle. Despite the difficulties with chromosome alignment, these cells exit mitosis because AurA overexpression also inactivates the spindle-assembly checkpoint. The cells ultimately fail in cytokinesis, producing tetraploid progeny. If the cells also lack p53, they continue through subsequent cell cycles, ultimately becoming polyploid and, eventually, aneuploid, with amplified centrosomes. AurA could also potentially contribute to carcinogenesis in other ways that are not shown here. Dysregulation of AurB might also lead to aneuploidy and cancer (lower left), but the mechanism is much less explored than that for AurA. (Adapted from Carmena and Earnshaw, 2003)

AurA overexpression has also been associated with increased cell proliferation and survival in response to cisplatin and UV (Wang et al., 2006). This has been shown to occur due to evasion of apoptosis via upregulation of Bcl-2, an apoptotic antagonist. Another possible mechanism shown for AurA overexpression-induced tumorigenesis is via increased epithelial-to-mesenchymal transition (EMT) by direct phosphorylation of MAPK by AurA in nasopharyngeal cancer (Wan et al., 2008). AurA was also shown to be a downstream target of MAPK in pancreatic cancers, where MAPK induces AurA expression (Furukawa et al., 2006). This suggested a possibility of existence of a feedback loop between the two, where initial overexpression of AurA by MAPK leads to further activation of the latter by direct phosphorylation (Wan et al., 2008). Increased cell migration in laryngeal cancer was also attributed to AurA overexpression leading to increased Akt1 phosphorylation (Guan et al., 2007). AurA overexpression was also associated with hyperactivation of the MEK/ERK signaling pathway (Tseng et al., 2009). Recently, AurA overexpression was shown to enhance E2F1 transcription activity and protein stability. It also induced increase of the microRNA cluster *miR-17-92* through E2F1 transcription factor (He et al., 2010). A gene amplification and mRNA overexpression independent pathway is also known to play a role in AurA induced transformation. This occurs via stabilization of the protein by increased phosphorylation at the S51 residue (Kitajima et al., 2007).

Thus, AurA overexpression promotes aneuploidy by deregulating SAC and p53, it promotes tumor proliferation by facilitating increased cell survival due to interference with apoptotic pathways and by promoting increased transcription of genes and, finally, promotes EMT by hyperphosphorylation of MAPK. Certainly, more work will be needed to unravel other molecular mechanisms by which AurA can promote cellular transformation and tumorigenesis.

1.6.3.1. Role of AurA-TPX2 complex in cancer

A recent review in the field has reassessed the potential of AurA alone as an oncogene (Asteriti et al., 2010). The authors maintain that a clearly defined role of AurA in promoting tumorigenesis is controversial and that the studies so far conducted have

found alterations in other pathways as an outcome of AurA overexpression. They suggest a more direct role of AurA by assessing the potential of AurA-TPX2 complex deregulation in cancers. Several studies have found overexpression of TPX2 in human cancers (Warner et al., 2009), (Satow et al., 2010). Many tumors have also been found to overexpress both AurA and TPX2 providing evidence of AurA-TPX2 complex as a functional unit in cancer progression (Asteriti et al., 2010). Mechanistically, the presence of an AurA-TPX2 functional complex has been envisioned to promote tumorigenesis by the three outcomes illustrated in Fig. 23:

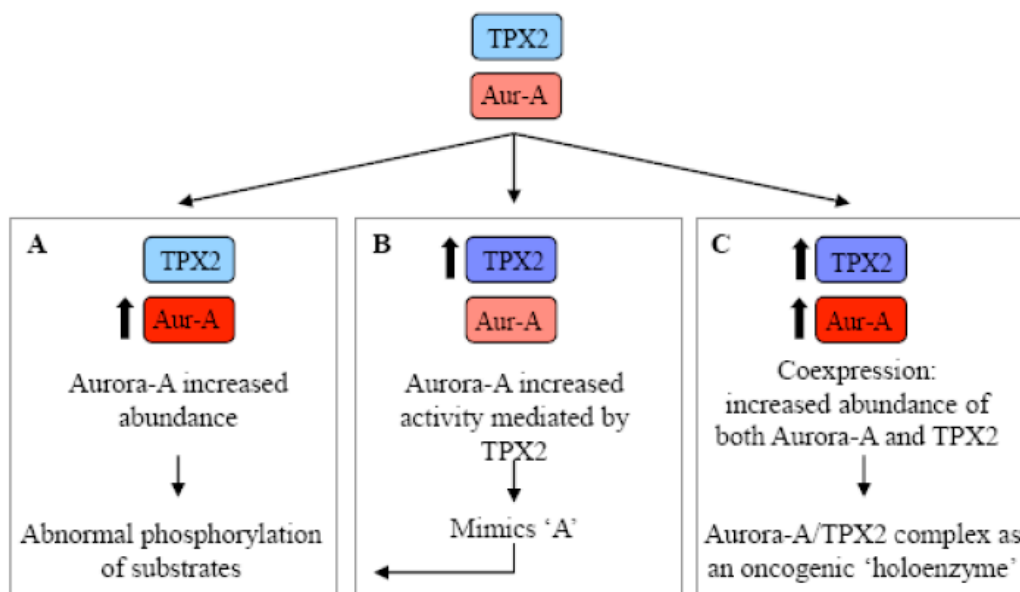


Figure 23- Possible outcomes of AurA-TPX2 deregulation in cancer progression. Increased AurA (A) or TPX2 (B) abundance, both expected to cause abnormal phosphorylation of AurA substrates, or overexpression of both (C); the latter scenario yields excessive abundance of the whole complex, which can then act as an oncogenic holoenzyme. (Adapted from Asteriti et al., 2010)

Recently, another study pointed to the role in cancer for an AurA mutant that is defective in binding TPX2 (Bibby et al., 2009), reinforcing the idea of the involvement of AurA regulators in facilitating tumorigenesis. Accordingly, in the study presented in this thesis, we observed loss of the TPX2-AurA interaction upon DNA damage, a mechanism that explains the inhibition of AurA activity detected in

response to genotoxic stress, in the face of unvaried protein level of the kinase. This may suggest that an unscheduled accumulation of AurA protein can occur in the absence of TPX2, making cells unresponsive to the presence of DNA damage, as they move on in mitosis with severe chromosome segregation defects. The fate of such aneuploid cells was, however, not assessed in the study presented in the thesis. Such an AurA overexpression-dependent insensitivity towards DNA damage has been shown previously (Wang et al., 2006). Moreover, it was shown that kinase activity is not required for AurA-mediated overriding of the SAC, suggesting a possible explanation of the role of accumulated AurA protein, which is devoid of kinase activity, in genetic instability (Jiang et al., 2003).

In conclusion, AurA-TPX2 complex deregulation could be one of the main pathways the alteration of which leads to tumorigenesis. It will be a great deal of interest for the future studies to understand the potential of this complex as a therapeutic target.

1.6.3.2. AurA as a chemotherapeutic target

As Aurora kinases are frequently found overexpressed in human cancers, they serve as attractive candidates of chemotherapeutic interventions. Downregulation of both AurA and B results in multiple mitotic defects and aneuploidy, ultimately leading to apoptosis. Many drugs targeting these kinases are currently in phase I and II clinical trials, but unfortunately, selectivity to AurA alone has not been successfully achieved so far. Drugs such as Hesperadin, MLN8054, MLN8237 are under phase I evaluation studies and have shown to result in phenotypes arising from inhibition of all three Aurora kinases, but show severe side effects (Kitzen et al., 2010).

VX-680 (MK-0457) so far is the most advanced Aurora inhibitor in clinical testing. It is currently under Phase I trial in patients with refractory leukemias and in Phase II trial with patients of advanced colorectal cancer (Miglarese and Carlson, 2006).

Table IV below provides information of drugs targeting the Aurora kinases.

Compound	Aurora A inhibition	Aurora B inhibition	Status	Comments
MK-0457 (VX-680)	+	+	Phase II	Also inhibits FLT3
MLN8054	+	—	Phase I—II	At high doses also inhibits Aurora B
SU6668	+	+	No longer in development	Also inhibits several receptor tyrosine kinases
Hesperadin	—	+	Pre-clinical	None
ZM447439	+	+	Phase I	Induces growth inhibition and apoptosis of human leukemia cells
PHA-680632	+	+	Pre-clinical	None
PHA-739358	+	+	Phase II	Proposed schedule: days 1, 8, and 15
AZD1152	—	+	Phase I	Synergistic action with tubulin depolymerizing agents and topoisomerase II inhibitors
JNJ-7706621	+	+	Pre-clinical	Also inhibits CDK1, CDK2, and CDK3
AT9283	+	+	Phase I	None
MP529	+	+	Pre-clinical	None
Resveratrol	+	+	Pre-clinical	Also inhibits Polo-like kinase 1
Rebamipide	Unknown	+	Clinical use in Japan	Inhibits Aurora B through survivin

Table IV- Aurora kinase inhibitors in clinical trials. (Adapted from Mountzios et al., 2008)

1.6.4. Role of AurA in regulation of translation

mRNA and protein level of several cell cycle proteins are highly regulated to ensure the proper timing of initiation and conclusion of cell cycle events. Translation of mRNA coding for genes required specifically during mitosis is one of such control mechanisms. Translational regulation, in addition to other factors, depends on the length of the poly(A) tail of the mRNA. Longer poly(A) tail promotes translation while shortening results in translation inhibition (Stutz et al., 1998). AurA is known to regulate the translation process via polyadenylation of mRNAs. AurA positively regulates *Xenopus* oocyte maturation in response to hormonal stimulation via phosphorylation of CPEB (cytoplasmic polyadenylation element binding protein) (Sarkissian et al., 2004). Phosphorylation of CPEB by AurA results in recruitment of CPSF (cleavage and polyadenylation specific factor) (Mendez et al., 2000) to promote polyadenylation at the 3' end of the mRNAs. In addition, it was postulated that an AurA-mediated polyadenylating activity in mitotic cell extracts is responsible for translation of cyclin B1 and Cdk1 mRNAs (Sasayama et al., 2005) and it was shown that cyclin B/Cdk1 controls translation of mitotic proteins by regulating their

recruitment to the polysomes, specifically during mitosis (Le Breton et al., 2003). In conclusion, AurA can modulate translation and hence provide an additional mechanism by which its overexpression can lead to mitotic abnormalities leading to tumorigenesis.

1.7. REGULATION OF AURORA A

1.7.1. Phosphorylation

Like other mitotic protein kinases, AurA is also regulated by reversible phosphorylation throughout mitosis. Three important sites of phosphorylation were mapped by subjecting recombinant AurA protein expressed in Sf9 cells and incubated with mitotic *Xenopus* extracts to mass spectrometric analysis (Littlepage et al., 2002). This identified S53, S349 (S51 and S342 in human AurA) and T295 (T288 in human AurA) as the major phosphorylated residues in AurA (Littlepage et al., 2002). T295/T288 resides in the catalytic domain of the kinase and phosphorylation of this residue results in increased kinase activity. It was initially shown that PKA could phosphorylate T288 *in vitro* and this resulted in increased AurA kinase activity (Walter et al., 2000) leading the authors to suggest that PKA may have a physiological role in the activation of AurA. Studies conducted elsewhere (Littlepage et al., 2002) and in our laboratory (Ferrari et al., 2005) disproved this claim, opening the possibility that other kinases could be involved in the process of AurA activation (Zhao et al., 2005). Also, since PKA activity is low during mitosis when AurA kinase activity peaks, this makes PKA less likely to activate AurA *in vivo* (Ferrari et al., 2005). Dephosphorylation of S53/S51 by PP2A, which belongs to ser/thr PP2 family, is required for the degradation of AurA (Horn et al., 2007), indicating that this site has an important role in the stability of AurA. The S349/S342 residue resides close to the PP1 binding motif in AurA. Recombinant AurA carrying mutation of S342 to Ala shows activity similar to the WT, whereas mutation of this residue to Asp completely abolishes enzymatic activity (Littlepage et al., 2002), (Ferrari S, unpublished data). Moreover, in *Xenopus*, phosphorylation of S349 caused reduction of AurA kinase activity and was shown to be required for the process of oocyte maturation (Pascreau et al., 2008). In contrast to these findings, S342 phosphorylation was found important for the kinase activity of AurA as the overexpressed S342A mutant from mitotic

COS-7 cells showed less than half of the kinase activity as that of WT when used on histone H3 as a substrate (Zhao et al., 2005). This shows that phosphorylation of S342 might be important for AurA kinase activity. Although a few *in vitro* data are available regarding the role of this residue in AurA regulation, no clear-cut evidence is present *in vivo* in human cells. Future work will be required to unequivocally establish the significance of S342 phosphorylation in AurA regulation.

1.7.2. Interaction partners

AurA interacts with several different proteins throughout G2 and mitosis that directly contribute to its phosphorylation, activation, localization, function and degradation. In this section I mention the most important proteins that are required for regulation of AurA.

1.7.2.1. General AurA interactors

1.7.2.1.1. p53

p53 is a known tumor suppressor required for preventing generation of transformed phenotypes by regulating processes of cell division and apoptosis (Chen et al., 2002). Most human tumors have mutated p53 status, implicating its effect in preventing cancer initiation/progression. p53 is also associated with the centrosome (Chen et al., 2002) and its downregulation results in centrosome amplification (Fukasawa et al., 1996), a phenotype significantly frequent in cells overexpressing AurA (Zhou et al., 1998). A direct relation between these two proteins in regulating centrosome amplification has been shown. Whereas AurA overexpression was shown to interfere with normal mitosis giving rise to multinucleation and centrosome amplification, deletion of p53 promoted cell cycle progression due to abrogation of the G1 checkpoint (Meraldi et al., 2002). p53 directly binds to N-terminal Aurora box in AurA and regulates its kinase activity in transactivation-independent manner (Chen et al., 2002). This interaction suppresses AurA kinase activity and also its ability to induce centrosome amplification. In addition to AurA being regulated by p53, AurA directly modulates the stability of p53 by phosphorylating it. AurA was shown to

phosphorylate p53 at S315 resulting in increased ubiquitination and degradation of p53 by Mdm2 (Katayama et al., 2004). Also, it was shown to phosphorylate p53 at S215 and interfere with its DNA binding and transactivation functions (Liu et al., 2004). Thus, deregulation of p53 stability and function by AurA-mediated phosphorylations has been suggested as a major route of cellular transformation resulting from AurA overexpression. The feedback regulation of the two proteins, therefore, significantly affects the functionality of cell cycle checkpoints.

1.7.2.1.2. Protein Phosphatase 1 (PP1)

AurA is bound to PP1 throughout the cell cycle but the binding is maximum during mitosis when AurA protein level peaks. AurA has two PP1-binding motifs (R/K)(V/I)XF: one includes the catalytic lysine residue (K₁₆₉VLF) and the second is immediately adjacent to S342 (K₃₄₃VEF) (Katayama et al., 2001). A feedback regulation exists between the two proteins, such that, the active AurA phosphorylates PP1 to promote its own activation while, if PP1 is active, it can dephosphorylate the kinase. This dephosphorylation is thought to be at the T288 site in AurA, although no direct *in vivo* evidence is available to support this claim. Control of the AurA-PP1 interaction may occur through phosphorylation at S342, an event that may decrease binding of PP1 to the K₃₄₃VEF motif, thereby maintaining T288 phosphorylated and, in turn, AurA active (Littlepage et al., 2002). This, however, still remains to be confirmed at both *in vitro* and *in vivo* levels. Interestingly, interaction with PP1 also seems to be indispensable for AurA as PP1 non-binding mutants of AurA are less active than the WT, suggesting that a balance between the phosphorylation/dephosphorylation states is finely tuned throughout the cell cycle (Katayama et al., 2001). The C-terminal region of PP1 inhibitor 2 (I-2) has also been shown to directly stimulate AurA kinase activity, at least *in vitro* (Satinover et al., 2004).

1.7.2.2. AurA interactors in G2

1.7.2.2.1. Bora

Bora, identified as an interactor of AurA, is highly conserved from *C.elegans* to humans. It is a nuclear protein that, upon Cdk1 activation, translocates to the cytoplasm and functions by interacting with AurA. It was initially identified due to its phenotypic similarity to AurA mutants and found to be a substrate and activator of the kinase (Hutterer et al., 2006). In subsequent studies, Bora was also found to activate Plk1 at the G2-M transition (Seki et al., 2008b). Bora is degraded at the end of G2 in Plk1 dependent manner through the β TrCP ubiquitin ligase (Seki et al., 2008a), thus allowing AurA to bind TPX2, its important mitotic regulator.

1.7.2.2.2. Ajuba

Ajuba is a LIM domain containing protein that was identified as an AurA interacting partner by a yeast two-hybrid screen (Hirota et al., 2003). LIM domain represents a double zinc-finger domain that was initially found in transcription factors like *C.elegans* Lin-11, rat Isl-1 and *C. elegans* mec-3 and therefore called LIM (Goyal et al., 1999). It was found to be promoting *Xenopus* oocyte maturation (Goyal et al., 1999). Ajuba interacts with AurA and is required for activation of the kinase at the centrosomes in late G2 as depletion of Ajuba results in absence of T288 phosphorylation of AurA and delay in mitotic entry (Hirota et al., 2003).

1.7.2.2.3. HEF1 (Human Enhancer of Filamentation 1)

HEF1 is a docking protein that plays an important role in controlling signaling at the focal adhesions (Pugacheva and Golemis, 2005). In addition, it also has a role in cell cycle regulation as it was found associating with the mitotic spindles (Law et al., 1998). Downregulation of HEF1 resulted in appearance of monopolar spindles while overexpression caused multipolar spindle formation (Pugacheva and Golemis, 2005), phenotypes similar to those arising from deregulated AurA levels. HEF1 interacts with AurA specifically during the G2 phase and allows activation of the kinase by

promoting autoactivation (Pugacheva and Golemis, 2005). AurA was found to be more active in cells overexpressing HEF1. HEF1 is also a substrate of AurA and is phosphorylated at S296 (Pugacheva and Golemis, 2005). Thus, in G2, HEF1 interaction activates AurA by increasing its autophosphorylation. As cells enter mitosis, phosphorylation of HEF1 at S296 by activated AurA causes dissociation of the complex. This provides a remarkable example of how regulation of signaling affects cell cycle progression.

1.7.2.2.4. PAK1 (p21 Activated Kinase 1)

The PAK family of protein kinases is a multifunctional class with roles in cell migration, cytoskeletal reorganization, focal adhesion and cell cycle regulation (Zhao et al., 2005). PAK1 localizes to the centrosomes where it interacts with AurA. It is the only kinase so far known to phosphorylate AurA at T288 and S342, an event that is necessary for AurA activation. Interaction of PAK1 with AurA at the centrosomes promotes centrosome duplication and cell cycle progression (Zhao et al., 2005).

1.7.2.3. AurA interactors in mitosis

1.7.2.3.1. TPX2

TPX2 (Targeting Protein for *Xenopus* kinesin-like protein 2) is a microtubule-associated protein first identified as the protein required for targeting Xklp2 (*Xenopus* kinesin-like protein 2) to the spindle poles (Wittmann et al., 1998). TPX2 was later found to interact with AurA and proved to be its substrate (Kufer et al., 2002). So far it remains the best-characterized AurA regulator.

Until mitosis begins, TPX2 is kept unavailable through binding to importin α and β . As soon as NEBD takes place, Ran-GTP mediates the release of TPX2, which is then free to bind AurA (Fig. 24) (Gruss and Vernos, 2004). Binding of TPX2 to AurA is mediated via its N-terminal domain (NTD) that interacts with C-terminal domain (CTD) of AurA and targets it to the spindle microtubules (Kufer et al., 2002). Within the NTD of TPX2, amino acids 1-43 are essential and sufficient for binding and

activating the kinase (Bayliss et al., 2003). Briefly, binding of TPX2 to AurA stretches its activation segment in a way that makes AurA competent to bind substrates and additionally protects the T-loop site T288 from PP1-dependent dephosphorylation (Bayliss et al., 2003). Mechanistically, binding of TPX2 pulls the activation segment such that the T288 moves into a buried position and becomes unavailable to the phosphatases. This, in addition, also exposes the nearby T287 residue that acts as a continuous substrate for the surrounding phosphatases, further protecting the T288 phosphorylation (Bayliss et al., 2003). Thus, TPX2 locks AurA in an active conformation. Active AurA, in turn, phosphorylates TPX2 and allows its spindle-associated functions to be carried out (Eyers and Maller, 2004), (Kufer et al., 2002). Recently, Plk1 was shown to promote activation of AurA *in vitro*, by directly phosphorylating TPX2 at S204 in *Xenopus* (Eckerdt et al., 2009). Furthermore, an AurA mutant associated with human tumors and unable to bind TPX2 was found to be mislocalized and misregulated (Bibby et al., 2009). Thus, interaction of AurA with TPX2 is essential for AurA activation and events such as chromatin-induced MT nucleation, spindle pole separation and for establishing proper spindle length.

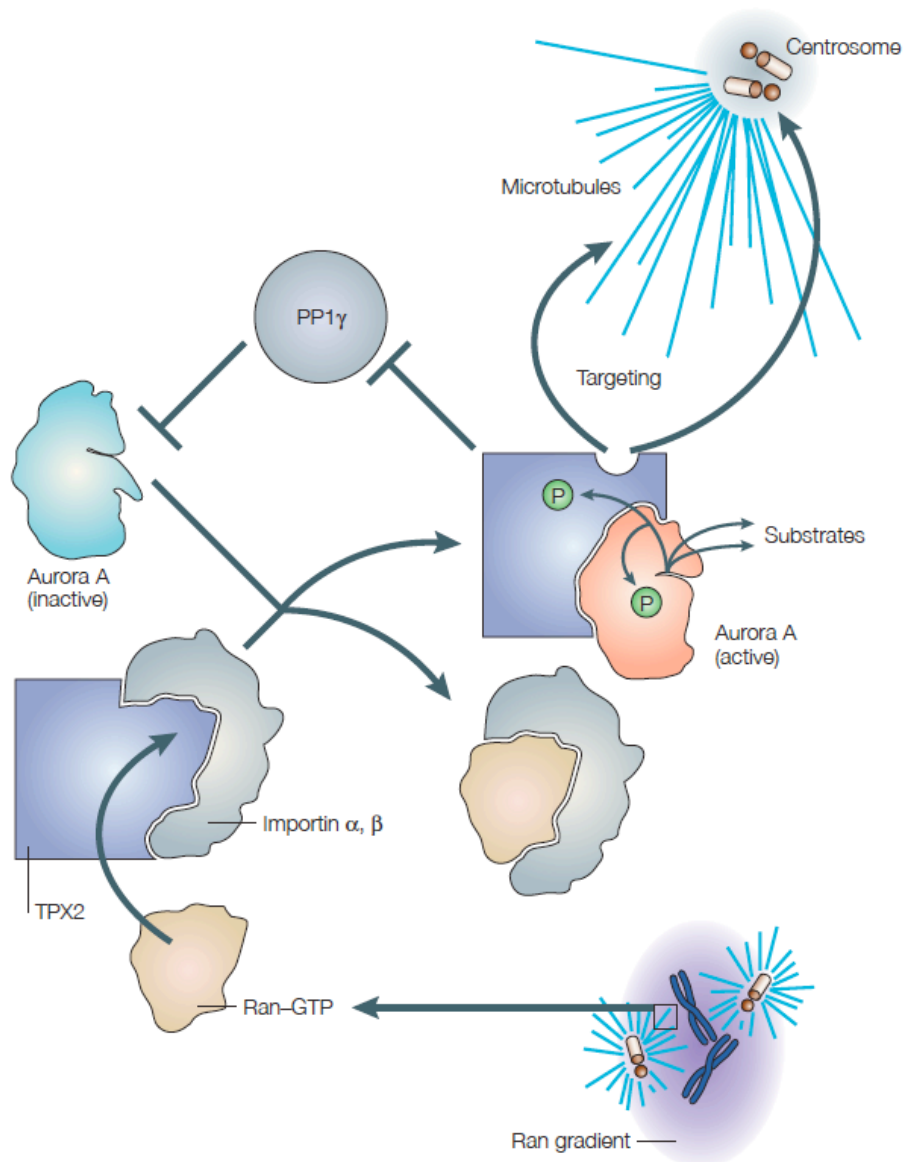


Figure 24- Regulation of AurA activity by TPX2 and Ran-GTP. As cells enter mitosis, TPX2 is in a complex with importins α or β . A gradient of Ran-GTP surrounding chromosomes (lower right) promotes the release of TPX2 from the importin. TPX2 then binds to AurA, which has been kept inactive by PP1. TPX2 interferes with PP1 action, enabling the kinase to autophosphorylate and activate itself and other substrates, including TPX2. TPX2 then also targets the kinase to microtubules proximal to the centrosome. (Adapted from Carmena and Earnshaw, 2003)

1.7.2.3.2. Astrin

Astrin was identified as a microtubule associating non-motor protein by mass-spectrometry performed on microtubules that were extracted from mammalian cells (Mack and Compton, 2001). It was found to interact with AurA by a yeast two-hybrid screen (Du et al., 2008). Astrin binding to AurA regulates its localization at the spindle microtubules, as depletion of astrin caused complete dissociation of AurA from spindles while the centrosomal localization was unaffected (Du et al., 2008). Depletion of astrin also caused an increase in the formation of multipolar spindles, possibly through misregulation of AurA (Du et al., 2008). Thus, apart from the known mitotic regulator of AurA, TPX2, that also controls its localization, astrin represents yet another protein directly influencing the localization and function of AurA.

1.7.2.3.3. RASSF1A (Ras Association Domain Family 1A)

RASSF1A is a potential tumor suppressor protein found mutated in most human cancers and may mediate its tumor suppressing activity either by promoting apoptosis or interfering with cell cycle progression (Agathangelou et al., 2005), (Dammann et al., 2005). It is primarily centrosomal localized in interphase (Guo et al., 2007) and relocalizes to the mitotic spindle as cells enter mitosis (Liu et al., 2008). AurA interacts with RASSF1A at the centrosomes and mitotic spindles and this interaction results in activation of AurA (Liu et al., 2008). AurA localization or stability, however, was not affected by this interaction (Liu et al., 2008). The exact functional significance of this requires further work.

Apart from these known interaction proteins, I could identify some novel AurA interactors during mitosis using proteomics approach. The outcome of this study is presented in detail in the Results Part 3.

1.7.3. Degradation of AurA

1.7.3.1. Role of APC/C complex

Like many other cell cycle regulated proteins, AurA is degraded at the end of mitosis through ubiquitin-dependent proteolysis via the proteasome pathway. Initial studies of this mechanism identified the APC/C complex as key player (Honda et al., 2000), (Walter et al., 2000) with the Cdc20 subunit being able to bind AurA (Farruggio et al., 1999). Later on, however, the direct involvement of Cdh1 in the process of AurA degradation was demonstrated, thus ruling out any roles for Cdc20 (Taguchi et al., 2002). Proteins that are degraded by Cdh1 possess special recognition motifs like an RxxL containing D-box (destruction box) and a KEN-box (composed of amino acids K-E-N). Human AurA has one D-box stretch in the NTD and four similar stretches in the kinase domain (none of them being a functional destruction box) (Honda et al., 2000) and a KEN-motif. In *Xenopus* and human AurA it was shown that destruction of AurA does not require the KEN-motif but requires the C-terminal D-box (Arlot-Bonnemains et al., 2001), (Castro et al., 2002), (Crane et al., 2004), a short N-terminus region called the A-box (Littlepage and Ruderman, 2002) and dephosphorylation of S51 residue in the A-box (Littlepage and Ruderman, 2002). Phosphorylation of S51 renders the kinase stable and dephosphorylation by PP2A is required for its destruction at the end of mitosis (Horn et al., 2007).

In addition, AurA destruction is also controlled by Plk1. Phosphorylation mediated activation of Cdc14A phosphatase by Plk1 leads to dephosphorylation and activation of Cdh1 at the end of mitosis, allowing AurA degradation (van Leuken et al., 2009). Thus in addition to regulating other processes of the cell cycle, phosphorylation also plays a key role in the destruction of key mitotic players during and at the end of mitosis through timely activation of either Cdc20 or Cdh1, respectively.

1.7.3.2. Role of CHFR E3 ligase

CHFR has been shown to prevent chromosomal instability by directly regulating AurA protein level (Yu et al., 2005). As a part of SAC, it was shown to delay mitotic

progression, an event that was accompanied by the presence of inactive AurA at centrosomes (Summers et al., 2005). CHFR can bind AurA through its C-terminal cysteine-rich region and this leads to ubiquitination of AurA (Yu et al., 2005). In *Chfr*-null MEFs, AurA overexpression-induced tumorigenesis was observed. Similar results were recently confirmed in human MCF10A cells where siRNA mediated downregulation of CHFR resulted in increased AurA protein level, followed by promotion of centrosome amplification (Privette and Petty, 2008). These data suggest that AurA stability is strictly monitored by more than one E3 ligase, indicating the importance of maintaining regulated AurA protein level to prevent chromosome instability.

1.7.3.3. Alternative AurA degradation pathway

In addition to the well-known ubiquitin-dependent proteasome degradation of AurA, an alternative ubiquitin-independent pathway is also involved in AurA destruction. AurA interacts with AurA kinase interacting protein 1 (AURKIP1), a negative regulator of the kinase and antizyme1 (Az1), member of the ubiquitin-independent degradation pathway. When in complex with AurA, AURKIP1 prevents polyubiquitination of the kinase and facilitates binding to Az1, thus promoting its degradation by the proteasome in an ubiquitin and cell-cycle independent manner (Lim and Gopalan, 2007b), (Lim and Gopalan, 2007a).

1.7.4. AURORA A AND DNA DAMAGE

1.7.4.1. Role of G2 checkpoint in regulating AurA

Apart from being regulated by a myriad of proteins in G2 and during mitosis, AurA is also regulated by DNA damage induced in G2 phase of the cell cycle. Three reports until now confirm this finding.

The first among these suggested that AurA activity decreased by damage induced G2 checkpoint in a cyclin B/Cdk1-dependent manner (Marumoto et al., 2002). The authors showed that overexpression of AurA could lead to bypass of this checkpoint

and cause premature entry into mitosis. Another study showed that upon DNA damage AurA was not activated and did not phosphorylate Cdc25B at S353 (Cazales et al., 2005). It was also shown that ectopic expression of AurA resulted in bypass of the checkpoint that was Chk1 dependent as the use of Chk1 inhibitor UCN-01 resulted in activation of AurA and hence phosphorylation of Cdc25B on S353. Previous work from my lab also showed that AurA is a target of the G2 checkpoint (Krystyniak et al., 2006). Inhibition of the kinase occurs in a Chk1-dependent manner, although Chk1 does not directly phosphorylate AurA. Inhibition of AurA was shown to cause accumulation of cells in G2, while reintroducing the WT kinase, but not the KD mutant, bypassed this arrest. This bypass was shown to be due to reactivation of Cdk1, placing Cdk1 downstream of AurA.

These results similarly concluded that AurA is a *bona fide* target of the G2 checkpoint and its inactivation prevents premature entry into mitosis.

1.7.4.2. AurA regulation by mitotic DNA damage

My thesis work was dedicated to extend the observations on DNA damage-dependent control of AurA to the M-phase of the cell cycle, where nothing is known about the regulation of AurA by genotoxic damage. The main aim was to understand if AurA is also a target of mitotic DNA damage and, if this is the case, what could be the underlying mechanism of its regulation. Details of this work are presented in the Results section (Part 1 and 2).

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2. BOOK CHAPTER

2.1. Role of cell cycle controllers in the development of cancer

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Role of cell cycle controllers in the development of cancer

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ABSTRACT

In this chapter we introduce proteins controlling transition through the cell division cycle, with particular focus on the controllers of mitosis, and we describe their role in the development of cancer.

The essay begins with a historical perspective on the discoveries that led to the formulation of “The Cell Theory” and the understanding of mechanisms controlling cell cycle transitions. This is followed by an in-depth description of pathways that monitor the appropriate completion of events in each phase of the cell division cycle (the checkpoints) and an analysis of the many dysfunctions that alter these mechanisms and account for the occurrence of cancer. The essay is concluded by some considerations on the opportunity of putting effort, both in basic research and drug discovery programs, on the full clarification of the G2/M checkpoint, which is the only checkpoint that tumors in the body and experimental systems have in common. The rationale to this suggestion is that combinations of low-dose radio- or chemotherapy and selective G2/M checkpoint inhibitors will be more effective in achieving tumor clearance than current conventional protocols.

1. INTRODUCTION: HISTORICAL PERSPECTIVE

1.1 The Cell Theory

Much like the introduction of first reflecting telescope opened the way to the discovery of the immensely far, so the early microscopes facilitated initial inspections of the extremely small. Using a prototype microscope to observe slices of cork bark, in 1665 the British physicist Robert Hooke first reported on structures that he named “cells” [1,2] (see also: <http://www.roberthooke.org.uk/>). It was, however, not before the technical improvement of microscopes, allowing for correction of chromatic aberration, and the advance of tissue preparation techniques that the German botanist Matthias Schleiden and the physiologist Theodor Schwann in 1838 and 1839, respectively, enunciated the “Cell Theory”. The essential concept of the Cell Theory is that cells are the basic units of all living organisms. In 1852 the pathologist Robert Remak, examining the behavior of frog eggs, reported that cells give rise to new cells through a process of cleavage that always starts with the nucleus [2]. This concept was further developed and elaborated by the Polish/German pathologist Rudolph Virchow, who proposed that every cell derives from a pre-existing cell in a continuous series of generations and is known for the aphorism “*Omnis cellula e cellula*” that he borrowed from the French chemist François Vincent Raspail (<http://www.britannica.com/eb/article-7719/Rudolf-Virchow>). The findings of Virchow and his contemporaries not only contributed to definitely dismiss the principle of spontaneous formation of organisms but also introduced the concept that diseases are likely explainable through alterations of the physiological state of cells, thus introducing the concept of cellular pathology. The subsequent identification of cellular organelles and subcellular compartments, which was rendered possible towards the end of the nineteenth century by improvements in tissue fixation and staining, expanded the notion that cells are merely composed of nucleus and protoplasm, thus opening the way to a functional description of cells as we know them today [1].

1.2 Cell Fusion Experiments

Knowledge of the cell’s architecture prompted studies aimed at understanding the internal wiring of cells and the mechanisms controlling their life and propagation.

In 1847 Schwann observed that when cells are close to each other their walls tend to coalesce but nuclei remain separated [3]. Such multi-nucleated cells (*heterokaryon*) are known as syncytia and their formation occurs both during development and in the mature organism [3]. Alternatively, cell fusion may be followed by nuclear fusion, in which case the deriving cell is a *synkaryon* [3].

The plasmodium *Physarum polycephalum* was used as model system in early cell fusion studies due to its ability of forming syncytia during its life cycle. Rush and coworkers noted that fusion of microplasmodia, each exhibiting synchronous mitosis but lacking synchrony in division with respect to each other, resulted in a large plasmodium in which the first mitosis occurred synchronously. This led the authors to postulate the existence of factors that are able to advance cells to mitosis [4]. At the same time, improvement of mammalian cell culturing techniques rendered possible the generation of artificial heterokaryons in the laboratory [5]. Using Sendai virus-mediated fusion of cells, Rao and Johnson conducted pioneering studies aimed at assessing the inter-dependence of cell cycle transitions. Specifically, the authors addressed the mechanism controlling DNA synthesis and mitosis in mammalian cells [6,7]. To this end, they fused cells that were either in the same stage or in different stages of the cell division cycle and observed the extent to which transition through the cycle was altered. Fusing S-phase with G1-phase cells in ratio 1:1 would drive the G1 nucleus to initiate DNA synthesis within 2 hrs from fusion, as compared to the 12 hrs needed by G1/G1 fused cells to initiate DNA replication [6]. Similarly, upon fusion of mitotic cells with a G1, an S or a G2 population of cells the authors observed the rapid occurrence of interphase chromatin condensation, a phenomenon referred as *premature chromosome condensation* or PCC [7]. Finally, fusion of S-phase cells with a G2 population did not induce re-replication of DNA in the G2 cells. The authors noted that all these effects were dosage-dependent, in the sense that the observed rate of fusion-induced progression to the next phase of the cell cycle was directly proportional to the number of most advanced cells used for the fusion. Together with the observations made in *Physarum*, the implication of these studies to the understanding of the regulation of cell division was enormous: in essence, the evidence provided by Rao and Johnson led to the concept that factors promoting entry into S- or M-phase must exist in cells that are about to replicate their genome or to divide, respectively. Such factors, in turn, act in a context-independent manner, in the

sense that once they are triggered they execute cell cycle transitions regardless of whether the cell is ready to perform such action.

1.3 Regulation of cell cycle transitions

The following years witnessed the convergence of genetic and biochemical studies that made use of different model systems to identify components of the machinery controlling cell cycle transitions. Pioneering investigation on the yeast *S. cerevisiae* conducted by Hartwell and collaborators led to the discovery of a number of new genes responsible for the execution of various steps of mitosis [8]. On the other hand, Nurse and colleagues identified 27 recessive mutants in *S. pombe* that were unable to complete the cell division cycle at restrictive temperature: 14 such genes were shown to participate in DNA synthesis, nuclear division and cell plate formation [9]. These studies led the authors to conclude that DNA synthesis and nuclear division form a cycle of mutually dependent events (for a detailed account of those studies see [10]). Despite the significant contribution of these investigations to the elucidation of the network of genes that control key cell cycle transitions, genetic approaches could not elucidate the mechanism by which such transitions are executed. It was only through biochemical analysis of protein function that molecular mechanisms could be understood. Initial studies performed on extracts of the plasmodium *Physarium* revealed that the increased activity of a protein kinase that was able to phosphorylate histone H1 correlated with entry into mitosis. Of particular notice, the authors observed that addition of the purified H1 kinase to plasmodia accelerated the initiation of mitosis [11].

On another front, experiments conducted on the frog *Rana pipiens* showed that injection into immature oocytes of the cytoplasm obtained from oocytes that were artificially induced to mature by administration of progesterone, led the former to mature and divide. Such substance was initially indicated as “Maturation Promoting Factor” [12] and later re-named M-phase Promoting Factor or MPF. Initial biochemical characterization of such factor showed that it consisted of a heat-labile protein [13]. This was later purified and demonstrated to possess protein kinase activity [14], in agreement with the evidence obtained from *Physarum*. The key contribution of Hunt’s laboratory to the field came from serendipitous observations made using the eggs of the sea urchin *Arbacia*. It was known that fertilization of eggs

correlates with increase in the rate of synthesis of proteins for which maternal mRNA provides the source. Hunt and collaborators detected a protein that was regularly synthesized and degraded in a manner that corresponded to transition through the phases of the cell division cycle: this protein was named “Cyclin” [15]. Subsequent work from a number of laboratories led to the identification of all other members of the cyclin-family and, most importantly, to the demonstration that cyclins are the regulatory subunits of cyclin-dependent kinases (CDKs) [10]. Our current understanding of the role of cyclins, cyclin-dependent kinases and their inhibitors (CKIs) is schematically illustrated in Fig. 1.

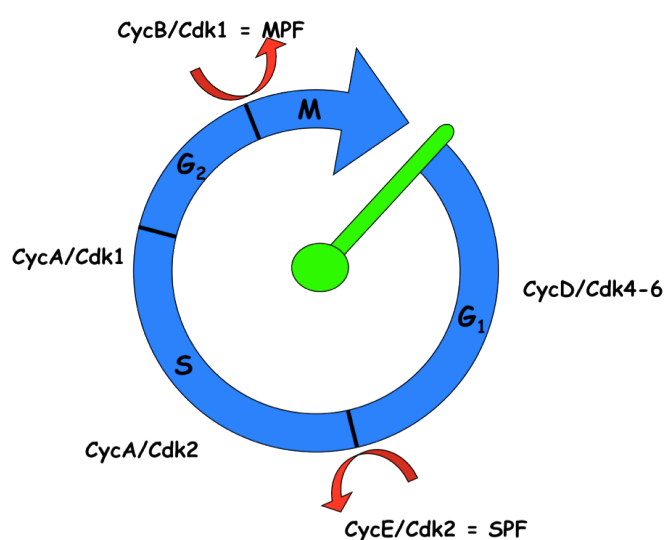


Figure 1. Regulation of cell cycle transitions.

Transition through the cell cycle is orchestrated by protein complexes constituted by regulatory (the cyclins, Cyc) and catalytic subunits (the cyclin-dependent kinases, CDKs). The two major transitions, consisting of entry into S-phase and into Mitosis, respectively, are controlled by the S-phase promoting factor (SPF) and the M-phase promoting factor (MPF) (see text for details). The time of action of Cyclin-dependent kinase inhibitors (CKIs, depicted within the circle) is also shown.

2. CELL CYCLE CONTROLLERS AS ONCOGENES

Considering both the complexity of the pathways controlling transition through the cell division cycle and the variety of proteins involved in this process, it is evident

that failure of a single wheel at any point in this mechanism may either impair or accelerate growth. Since extensive literature on the deregulation of classic cell cycle controllers (i.e., cyclins, CDKs and CKIs) and their targets (pRb and p53) already exists, here we will provide a brief account of the most significant work in this field and will rather concentrate to discuss some of the most intriguing pathways that operate at the onset of mitosis and are involved with the development of cancer.

Early studies in mouse model systems showed that over-expression of Cyclin D1 and E1 led to hyperplasia and to the development of carcinomas [16,17], suggesting that both cyclins might be oncogenes. However, it was later observed that ablation of Cyclin D1 [18,19] as well as inactivation of the partner subunits Cdk4 or Cdk6 [20-22] was not sufficient to block progression through the cell cycle, pointing to the existence of redundant pathways. Similarly, Cyclin B1 knock-out did not affect early development, with embryos reaching mid-gestation, whereas Cyclin B2-null mice showed no phenotype at all [23]. Somehow more severe was the phenotype resulting from Cyclin A2 ablation, where embryogenesis could not go over the blastula stage [24]. Lack of the Cyclin A partner Cdk2 did not appear to affect viability of the animals [25], likely due to the compensating role played by Cdk1 [26], whereas Cdk4/Cdk2 double knock-out resulted to be embryonic lethal [27]. Once these compensatory functions began to be appreciated, it appeared clear that only the generation of compound knock-out mice would have helped clarifying the overlapping roles of Cyclins and Cdk (for a review see [28]).

Substantially, the same trend was observed upon ablation of individual Cyclin-dependent kinase-inhibitors [28]. In all cases, with the notable exception of p57^{Kip2} [29,30], lack of CKIs expression did not affect viability of the animals but, interestingly, rendered them prone to the development of cancer (reviewed in [28]).

Analysis of human tumors revealed that the most common alterations affect controllers of the G1/S transition, encompassing overexpression of cyclins (D1 and E1) and CDKs (CDK4 and CDK6) as well as loss of CKI (INK4A, INK4B and KIP1). Genetic analysis showed that such alterations occurred at the chromosomal level, with frequent amplification of cyclin D1 or CDK4, translocation of CDK6 and deletions of INK4 genes. Cases of epigenetic inactivation of INK4 were also reported [31].

3. MITOSIS

Mitosis is a highly regulated process that ensures equal distribution of the duplicated genome to the daughter nuclei, thus allowing new generations of cells to inherit a complete set of genetic information. Mitosis comprises five phases consisting of prophase (initial chromosome condensation and nuclear envelope breakdown), prometaphase (association of condensed chromatids with fibers of the mitotic spindle), metaphase (complete attachment of chromatids to spindle microtubules and congression to the metaphase plate), anaphase (sister-chromatid separation) and telophase (chromosome decondensation and reformation of the nuclear envelope), which is followed by cytokinesis. Accurate transmission of genetic information requires the mechanisms controlling entry, transition through, and exit from mitosis to operate with great precision. Since cancer is a disease characterized by uncontrolled proliferation, genomic instability and aneuploidy, deregulation of the mechanisms controlling cell division greatly enhances the chance of occurrence of a tumor. Checkpoints are control mechanisms that operate during progression through the cell cycle and are set in place to prevent transition from one phase to the next until certain conditions are satisfied [32]. Whereas the G1/S checkpoint is often lost in cancer cells as result of loss of p53 function, the checkpoints controlling the onset and the transition through mitosis are fully functional in every cell type so far examined [33]. The major point of control put in place prior to mitosis is the G2 checkpoint, whereas the spindle-assembly checkpoint operates during mitosis. The G2 checkpoint is in charge of controlling the completion of DNA replication as well as the status and the topology (i.e., catenation) of the duplicated genetic material. The mitotic checkpoint delays the metaphase-to-anaphase transition in response to improper kinetochore-microtubule attachment and tension at the site of attachment.

In the next sections we will dissect the mechanism of action of the checkpoints mentioned above and we will highlight the defects that contribute to tumorigenesis.

3.1 The G2 checkpoint

Definition of “G2 checkpoint” given here conglomerates a number of control mechanisms that are put in place by the cell to ensure fidelity and accuracy of the steps preceding cell division and that are necessary to the correct execution of mitosis. Some of the pathways that we have grouped under the definition “G2-checkpoint”

have been proposed by others as genuine “checkpoints” *per se*. This tendency has led to the unnecessary proliferation of possible points of control during progression to mitosis. We will present evidence indicating that such pathways are rather “subroutines” of the same program.

3.1.1 DNA damage

Integrity of the genome is the first condition to the survival of all organisms. Damage to DNA is a continuous threat posed by the intrinsic inaccuracy of the DNA replication machinery, by the oxidative environment in which we live as well as by the genotoxic stress that is caused by inhaled cigarette smoke, ultraviolet light and dietary factors. In order to maintain genomic stability, a network of proteins has evolved with the function of sensing and repairing DNA damage. Processing of different lesions that result from the distinct mechanism of action of genotoxic agents is accompanied by the generation of signals that delay the onset of mitosis [34]. This is known as the DNA damage response (DDR) [35]. To the purpose of this discussion, we will examine how signals that originate at sites of DNA damage impinge onto the cell cycle machinery, and we refer the reader to specialized literature for an in-depth view of mechanism of DNA repair [36].

The upstream signaling components of the DDR pathway are ATM (Ataxia telangiectasia-mutated) and ATR (ATM- and Rad3-related), two members of the PI3K (phosphatidyl-inositol-3-OH kinase) family. The common view attributes to ATM the ability of detecting and coordinating initial processing of physical damage to DNA (particularly double strand breaks), whereas ATR is preferentially involved in the response to DNA replication stress [35]. However, since initial processing of damaged DNA leads to the generation of structures similar to those produced during DNA replication, concomitant activation of ATM and ATR has been reported at sites of damage [37]. ATM/ATR-dependent phosphorylation of the downstream checkpoint kinases 1 and 2 (CHK1 and CHK2) allows transducing in a rapid and consistent manner the damage signal to effector-proteins that control DNA repair, cell cycle progression and apoptosis [38]. As we learned from the scheme presented in Fig.1, the master regulator of the G2/M transition is CycB/CDK1. One branch of the DDR negatively regulates CycB/CDK1 through activation of the WEE1 kinase and inhibition of the CDC25 phosphatases. Briefly, signals from damaged DNA (as well

as from incomplete DNA replication) impinge on WEE1 through checkpoint kinases [39] and result in the phosphorylation of two negative sites in CDK1 ATP-binding domain, in a manner that blocks its catalytic activity (reviewed in [40]). In parallel, CHK1/CHK2 driven phosphorylation of CDC25C, the phosphatase responsible for dephosphorylation of the negative sites in CDK1 ATP-binding domain, leads to 14-3-3-mediated nuclear export of the phosphatase [41,42], thus blocking its function. This two-pronged pathway [43], by converging on the same target, contributes to hold CDK1 in check for the time that DNA damage is being addressed. In addition to inhibition of CycB/CDK1, inactivation of other mitotic kinases operating upstream or in parallel to CycB/CDK1, like Aurora A [44] and the Polo-like kinase Plk1 [45], is an important determinant of the DNA damage-induced G2/M arrest (reviewed in [40]).

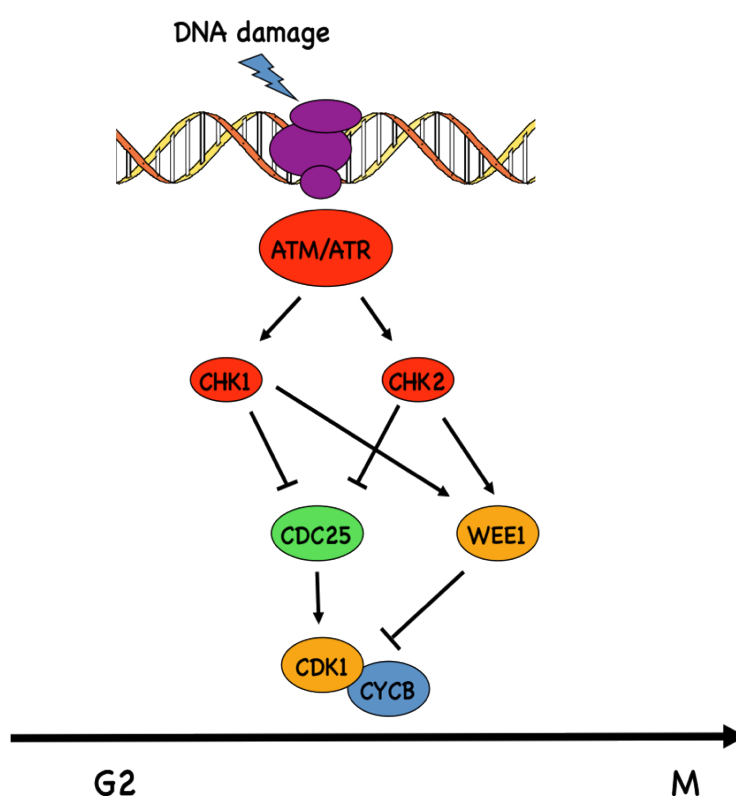


Figure 2. The DNA damage response.

DNA damage caused by genotoxic agents is initially detected by dedicated protein complexes (the sensors, grey ovals) that facilitate initial processing of the damage and recruitment of protein kinases of the PIKK family (ATM/ATR). The latter trigger a phosphorylation cascade that targets checkpoint kinases (CHK1/CHK2) as well as the

tumor suppressor p53 (not shown). CHK1- and CHK2-dependent phosphorylation of WEE1 and CDC25, as well as inactivation of Aurora-A leads to inhibition of CDK1 and G2/M arrest (see text for details).

The impact of a defective or loose DDR to the development of cancer is exemplified by a number of syndromes that, among the many dysfunctions presented by the patients affected by such syndromes, are characterized by cancer predisposition. This is the case of Ataxia telangiectasia (AT), a disorder caused by mutations of the gene encoding ATM [46] and Nijmegen Breakage Syndrome, where the NBS1 component of the MRE11-RAD50-NBS1 complex that is responsible for activation of ATM is deficient [47]. ATLD is a rare disorder caused by hypomorphic mutations of MRE11 component of the MRN complex with patients displaying similar symptoms of AT patients [48]. Hypomorphic mutations in Artemis, a protein involved in the pathway addressing repair of double-strand breaks, lead to predisposition to lymphomas [49]. Mutation of the familial breast cancer genes *BRCA1* or *BRCA2*, which are important mediators of signals in the DDR, predispose to breast and ovarian cancer [50]. Fanconi anemia is an multigenic autosomal recessive condition where patients display hypersensitivity to interstrand DNA crosslinks due to defects in the coordination of repair [51]. Fanconi patients display increased incidence of solid tumors as well as leukemia [52,53]. Another autosomal recessive disease, Xeroderma pigmentosum (XP), is characterized by defective nucleotide excision repair (NER) of DNA damage caused by UV-radiation and the early onset of skin cancer [54]. Predisposition to sarcoma, breast cancer and brain tumors was described in Li-Fraumeni Syndrome patients, who carry inherited mutations in p53 and heterozygous germ line mutations of the *CHK2* gene [55]. Bloom Syndrome patients carry mutations in the *BLM* gene that codes for a helicase necessary to the maintenance of genome stability [56]. *BLM* mutations increase the risk of colorectal cancer [57]. Germline defects in one of the four genes encoding for proteins that correct base mismatches in DNA (mismatch repair or MMR) lead to inactivation of this pathway and is the hallmark of hereditary non-polyposis colorectal cancer (HNPCC) [58].

Over-expression of essential mitotic kinases was proposed to result in facilitated bypass of the DNA damage-induced G2/M checkpoint and, in turn, to promote

tumorigenesis. This is the case of NEK2 [59], a kinase that regulates centrosome separation [60], PLK1 [61], a kinase that controls centrosome maturation and the activity of the APC/C among other multiple aspects of mitosis [40] and Aurora A [62-64], a kinase that controls ploidy [40].

In conclusion, defects in the pathways responsible for DNA damage recognition, DNA repair as well as the associated signaling, lead to the development of cancer. The latter appears to be a consequence of the fact that mutations in key components of such pathways impair the ability of cells to arrest before division, thus allowing the progressive accumulation of damage in the genome.

3.1.2 DNA decatenation

Catenations in DNA typically arise after replication or occur accidentally during interphase. Resolving entanglements is absolutely essential to prevent chromosomal missegregations and aneuploidy. This process, known as decatenation, is mediated by topoisomerases of the class II subtype that, in a two-step reaction, cleave and re-close pairs of complementary DNA strands before the onset of mitosis [65]. Failure to decatenate chromatids prevents entry into mitosis until DNA entanglements have been resolved [66]. Whether this triggers a checkpoint is controversial, since cells normally reach mitosis with chromatids still entangled at the centromeric region [67]. Early studies suggested the decatenation checkpoint to be distinct from the G2 DNA damage checkpoint [66]. Such conclusion was based on the comparison of the effects of catalytic inhibitors (ICRF-193) and classic poisons of topoisomerase II (etoposide). Whereas the former inhibit different steps of the catalytic process of DNA incision, as for instance the intrinsic ATPase activity of topoisomerase II, thus sequestering the enzyme from turnover [68,69], the latter bind and stabilize the enzyme-DNA complex, thus exposing cleaved double-strand ends [69]. In the case of topoisomerase II poisons, it was observed that the massive amount of DNA damage produced by these compounds resulted in a strong activation of the ATM pathway [70]. On the contrary, the mitotic delay caused by catalytic inhibitors of topoisomerase II was initially shown to be ATM-independent, although it appeared to require ATR and BRCA1 [71]. Recently, however, a reassessment of this issue led to the conclusion that ICRF-193 induces ATM activation in human lung cancer cell lines [72] and this is accompanied by a fully-fledged DNA damage response [73].

In addition to the involvement of ATR, evidence that the RecQ helicase Werner (WRN) is also implicated in the decatenation checkpoint was derived from the observation that in the absence of a functional helicase, cells do not undergo mitotic delay in response to catalytic inhibitors of topoisomerase II [74].

Just like the failure of other checkpoints in the cell promotes carcinogenesis, loss of the mechanism controlling DNA decatenation is another major route to development of cancer [72,75].

3.2 The mitotic checkpoint

3.2.1 Microtubule network

Drugs targeting the microtubule network, such as nocodazole or taxol, affect processes that occur at the onset of mitosis like centrosome separation, alignment of chromosomes on the metaphase plate and separation of the sister chromatids at anaphase. This is associated with induction of stress responses in the cell.

The early-mitotic checkpoint, also known as Chfr-checkpoint (Checkpoint-protein with FHA and Ring-finger domain), operates in late G2. This pathway was initially described to selectively monitor the extent of centrosome separation [76], an event required for the formation of a bipolar mitotic spindle. However, selective inhibitors of centrosome separation that do not affect the overall architecture of the microtubule network, like monastrol [77], do not trigger the Chfr-checkpoint. Therefore, lack of centrosome separation is likely not the specific trigger of this pathway, which rather responds to perturbation of the microtubule network architecture.

The Chfr protein is a ubiquitin ligase that ubiquitinates PLK1 [78] and Aurora-A [79] thus blocking the cascade of events that result in CDK1 activation. Homozygous and heterozygous Chfr-knockout mice develop spontaneous and carcinogen-induced tumors, indicating that Chfr is a genuine tumor suppressor gene [79]. Moreover, cells derived from Chfr-null mice display aneuploidy, chromosomal segregation defects and cytokinesis failure [79]. The *CHFR* gene was found to be frequently inactivated in human cancers due to aberrant hypermethylation of the promoter [80,81].

3.2.2 Spindle Assembly

The Spindle Assembly Checkpoint (SAC) pathway includes three classes of proteins: Mitotic-Arrest Deficient (MAD), Budding Uninhibited by Benzimidazole (BUB) and

Monopolar Spindle (MPS). The corresponding genes (*MAD1*, *MAD2* and *MAD3*; *BUB1* and *BUB3* and *MPS1*) were identified in mutants of the budding yeast *S. cerevisiae* that were unable to arrest in mitosis in the presence of spindle poisons [82,83] (for a detailed description of the SAC pathway see [84]). Spindle poisons are drugs currently used in chemotherapy that either stabilize microtubules by directly binding to tubulin (taxanes and colchicine), thus preventing microtubule polymerization, or facilitate microtubules depolymerization (nocodazole), thereby preventing the formation of correct bipolar spindles. In the presence of spindle poisons, SAC proteins act to prevent entry and progression through mitosis. To understand this mechanism, we will take a short digression and explain the cascade of events that trigger anaphase.

In an unperturbed mitosis, SAC proteins are sequentially recruited to kinetochores of unattached chromosomes [85]. This results in the formation of the so-called Mitotic Checkpoint Complex (MCC) that includes the two complexes MAD1/MAD2 and BUB3/BUB-R1 (BUB-R1 being the human orthologue of *S. cerevisiae* Mad3). Both complexes are able to bind CDC20 and in this manner prevent its interaction with the APC/Cyclosome, a multi-subunit E3-ligase that initiates the degradation of CYC-B and securin. Upon stable attachment of all kinetochores to microtubules that have nucleated from opposite centrosomes (a phenomenon named bi-orientation), CDC20 is released from MCC and binds to APC/C. APC/C-dependent destruction of CYC-B impairs the activity of CDK1, whereas degradation of securin releases separase. The latter initiates the proteolysis of cohesins, proteins that hold chromatids together, thus triggering the transition from metaphase to anaphase [86]. In the case of lack of proper kinetochore-microtubule attachment or lack of tension at the site of attachment, release of CDC20 from MCC is blocked [84]. Control of MCC formation as well as amplification of the SAC signal is orchestrated by the protein kinases MPS1, Aurora-B, MAPK, PLK1, CYC-B/CDK1 and NEK2. The role of Aurora-B is paradigmatic of the case of merotelic attachment (i.e., microtubules originating from opposite poles that make contact with a single sister kinetochore): in this setting, persistent Aurora-B activity leads to release of attachment, in a manner that likely depends on phosphorylation of MCAK (mitotic centromere-associated kinesin), a protein that is able to depolymerize microtubules [87-89]. This, in turn, causes release of tension and prevents anaphase. Aurora-B overexpression has been documented in a

number of human cancers [90] and a recently developed inhibitor selective to this kinase has confirmed Aurora-B as interesting target in cancer therapy [91].

The first indication that gene mutations affecting the spindle checkpoint may result in aneuploidy was derived from the comparison of cells displaying either microsatellite instability (MIN) or chromosomal instability (CIN). Whereas the former present a normal spindle assembly checkpoint, the latter do not. The authors showed that expression of either of the two naturally occurring BUB1 mutants converted the normal checkpoint status of MIN cells to the defective one of CIN cells [92]. A study on five families presenting mosaic variegated aneuploidy, a rare human disorder [93], established the causal connection between BUB1B biallelic mutations and CIN in humans. Animal studies showed that transient MAD2 over-expression is sufficient for the initiation of tumorigenesis through the promotion of aneuploidy [94]. Accordingly, MAD2 over-expression has been reported in a number of human cancers [95] and shown to correlate with poor prognosis [96,97].

However, as shown for DNA damage, where the properties of genotoxic agents are exploited in therapeutic protocols despite the established link between DNA damage and mutagenesis [98], also in the case of the spindle checkpoint, processes conferring selective growth advantage to cancer cells may be their “Achilles’ heel”. In this respect, studies on human cancer cell lines have shown that suppression of the spindle assembly checkpoint, either through reduced expression of BUBR1 or MAD2 or through inhibition of BUBR1 kinase activity, was lethal due to massive chromosome loss [99]. This indicates that disabling SAC signaling is a feasible approach in anticancer therapy [100].

4. PERSPECTIVE

The dogma that radiation eliminates cancer cells by triggering apoptosis has prompted studies on the mechanism of action of DNA-damaging agents and the search for radiomimetic drugs. This dogma has been recently challenged by observations on the behavior of cancers that develop in the human body. The essence of the challenging argument is that solid cancers are unlikely to retain the property to self-destruct by apoptosis because it is precisely through inactivation of the apoptotic machinery and promotion of aggressive vascularization that cancer cells manage to recover from latency and form solid tumors. This suggests that radiation and drugs that cause tumor

shrinkage *in vivo* must operate by mechanisms other than apoptosis. Indeed, oncologists and radiation biologists have observed that in tumors undergoing radio- or chemotherapy, cells die when they try to divide and, typically, daughter chromosomes break when they attempt to separate during mitosis [101]. For this reason significant effort in basic research and drug discovery programs is put to the elucidation of the checkpoint that tumors in the body and experimental systems, like cultured cancer cells, have in common: this is the G2/M checkpoint.

It is believed that in the near future, treatment of cancer patients with a combination of tolerable dosages of conventional radio- or chemotherapy and selective drugs that inactivate the key cell cycle checkpoints will likely be the avenue to achieve clearance or at least shrinkage of solid tumors [33,100].

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3. AIM OF MY THESIS PROJECT(S)

3.1. AIM 1

Aurora A (AurA) is a protein kinase required for entry into mitosis and for mitotic progression. It was previously shown in my lab and by others that AurA is a target of the G2 checkpoint. Exposing cells to DNA damage in G2 causes inhibition of the kinase in a Chk1 dependent manner resulting in a prolonged arrest of cells in G2 and delay in mitotic entry. Reintroducing the WT but not the KD AurA mutant allowed cells to enter mitosis by reactivation of Cdk1. This study revealed that AurA is highly regulated by G2 DNA damage and placed AurA upstream of Cdk1.

The aim of my project was to extend this study further to understand a) regulation of AurA in response to mitotic DNA damage and b) study the fate of execution of mitosis after DNA damage.

3.2. AIM 2

The process of mitosis has been studied for more than two decades and still many secrets are yet to be revealed. AurA is just one of those many known (and unknown) proteins that ensure fidelity of mitosis. Many novel proteins are constantly being discovered that broaden our current view of these processes.

My second project aimed to identify novel interactors of AurA by mass spectrometry in order to gain a better understanding of the role of AurA during mitosis.

4. RESULTS

PART 1

Understanding the Mechanism of Regulation of Protein Kinase Aurora A in Response to Mitotic DNA Damage

4.1. Mitotic DNA damage targets the Aurora A/TPX2 complex

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ABSTRACT

We have previously shown that the DNA damage-induced G2 arrest is contributed by inhibition of Aurora A (AurA) and that transduction of active AurA into arrested cells allows bypassing the block through reactivation of CDK1. In this study, we investigated the mechanism of DNA damage-induced AurA inhibition. We provide evidence that ionizing radiation (IR) administered in mitosis, a time when AurA protein and enzymatic activity reach peak levels, impairs interaction with the partner TPX2, leading to inactivation of the kinase through dephosphorylation of AurA T-loop residue, T₂₈₈. We find that decreased AurA-TPX2 complex formation in response to irradiation results from reduced cellular levels of TPX2, an effect that is both contributed by increased APC/CDH1-dependent protein degradation and decreased translation of TPX2 mRNA.

Keywords: Aurora A, DNA damage, mitosis, TPX2, translation

INTRODUCTION

The genetic information contained in DNA is duplicated and faithfully segregated into daughter cells during a cycle of cell division. Errors in DNA replication or in the repair of damaged DNA underlie the process of carcinogenesis.¹ In normal cells, the status of DNA is continuously monitored by a myriad of proteins that are able to recognize distinct types of damage and address their repair. In parallel to repair, cells mount a response that slows down or arrests the cell cycle through the activation of signaling pathways impinging on cell cycle controllers.²

In response to incomplete DNA replication or DNA damage occurring in G2, progression to mitosis is arrested in an ATM/ATR-dependent manner.² In antepause, a time spanning from the conclusion of G2 to the initiation of chromosome condensation,^{3,4} cells respond to DNA damage⁵ or microtubule poisons⁶ through the activation of a checkpoint that is not mediated by PI-3K-like kinases. The execution of this checkpoint instead, depends on two proteins, the CHFR E3 ligase⁷ and the p38 MAPK.⁸ Cells containing a wild-type antepause checkpoint undergo chromosome decondensation and revert to a G2-like state,⁵ whereas cells lacking a functional CHFR progress into mitosis.⁹ Damage occurring upon completion of antepause does not normally cause reversion to an early stage of the cell cycle but rather triggers mitotic arrest through activation of the spindle assembly checkpoint (SAC),^{10,11} though exceptions have been reported.¹² Contrary to mammalian cells, mitotic DNA damage in *X. laevis* and DT40 cells prevents spindle assembly in an ATM/ATR-dependent manner.¹³ This was proposed as an additional pathway monitoring chromosome breaks that have escaped the G2/M checkpoint.

The Auroras are mitotic kinases first identified in yeast and *Drosophila* as regulators of centrosome separation, bipolar spindle formation, chromosome segregation and cytokinesis.¹⁴ Three human Aurora genes have been described, namely *Aurora A*, *B* and *C*. Aurora A (AurA) protein level and kinase activity peak at G2 and remain high throughout mitosis.^{15,16} The timing and extent of AurA activation is controlled by interaction with a variety of proteins. Interaction with protein phosphatase 1 (PP1) results in the reciprocal control of enzymatic activity,¹⁷ whereas binding to Bora in G2¹⁸ supports AurA activation. Accordingly, depletion of Bora causes insufficient AurA activation and multiple spindle defects.¹⁹ The PLK1-dependent degradation of

Bora, which is mediated by the β TrCP ubiquitin ligase,¹⁹ facilitates full AurA activation in mitosis through interaction with TPX2,²⁰ an event that is contributed by PLK1-mediated TPX2 phosphorylation.²¹ Interaction with TPX2 maintains the kinase in an active conformation by protecting the T-loop site T₂₈₈ from dephosphorylation.^{20,22} Another important component of the machinery controlling AurA activation seem to be the focal adhesion protein HEF1, which interacts with the centrosomal kinases AurA and Nek2 and supports their activation.²³ Finally, the protein kinase PAK, which localizes to centrosomes in mitosis, has been reported to phosphorylate and activate AurA.²⁴

To identify the molecular mechanism of AurA inhibition in response to genotoxic stress, we examined the effect of IR on mitotic cells, where AurA protein level and enzymatic activity is maximal. We found that IR led to a reduction of AurA/TPX2 complex. This was the result of decreased TPX2 protein expression, depending on both accelerated protein degradation as well as failure to recruit TPX2 mRNA to actively translating polysomes. As a consequence, deprotection of AurA T-loop site T₂₈₈ caused its rapid dephosphorylation. Although cells succeeded in completing mitosis under these conditions, they displayed severe chromosome segregation defects.

RESULTS

Inhibition of Aurora A kinase activity in response to mitotic DNA damage

We initially decided to examine the effect of DNA damage on AurA during transition through mitosis. Using HeLa cells synchronized by double-thymidine block (DTB) release (Fig. S1A and S1B), we observed that AurA protein reached highest levels in mitosis (9-10h), in a manner similar to that observed for Cyclin B1 (Fig. 1A).¹⁶ Furthermore, TPX2 appeared to be maximally expressed in mitosis (Fig. 1A). The enzymatic activity of AurA, measured using a model peptide substrate,²⁵ followed the pattern of protein expression, reaching a peak of activity when the expression of the activating partner TPX2 was maximal (Fig. S1C).

To assess the effect of genotoxic damage on mitotic AurA, synchronized cells were treated with nocodazole (200 ng/ml, 7h) and irradiated (9 Gy, 10h). Flow cytometric analysis showed that nocodazole effectively maintained cells in mitosis for the duration of the experiment (Fig. S1D). Despite the forced permanence in mitosis, IR caused a decrease in AurA kinase activity, as indicated by dephosphorylation of the T-loop site T₂₈₈ (Fig. 1B, 12h-14h).²⁵⁻²⁷ Under such conditions, AurA protein levels remained unchanged at the time points examined.

Mitotic DNA damage does not affect the completion of mitosis

In response to mitotic DNA damage, we observed rapid loss of the H3-pS₁₀ signal (Fig. 2A), indicative of chromatin decondensation. This could result from either reversal to a G2-like state or a premature exit from mitosis. To distinguish between these two possibilities, we examined a number of parameters. Flow cytometric analysis of the cell cycle distribution did not show any significant differences between control and irradiated cells (Fig. S2A). CDK1 phosphorylation at the inhibitory site Y₁₅, which is high in G2 cells (Fig. S2B, S2C), displayed a similar time-dependent decrease in untreated and irradiated cells (Fig. 2B), although it was incomplete in the latter. Time-lapse microscopy showed that cells in metaphase at the time of irradiation or 1h post-irradiation were equally able to complete mitotic division (Fig. S2D). Finally, we quantified early (pro- and metaphase) and late (ana- and telophase) mitotic figures in control or irradiated cells (Fig. 2C). Although at 1h post-irradiation the proportion of early vs. late mitotic figures was inverted as compared to controls,

likely due to the decreased entry into mitosis of cells that received irradiation while still at the G2/M transition, late mitotic figures were detectable at this time point. This indicates that irradiation during mitosis does not cause reversion to a G2-like state.

To assess the quality of the mitosis executed by irradiated cells, we stained DNA with DAPI. We found a significant increase in misaligned metaphase chromosomes after IR, as well as chromosome bridges and lagging chromosomes in anaphase (Fig. S3A-C). We also noticed that after cytokinesis, lagging chromosomes led to the formation of micronuclei to a significantly larger extent in irradiated cells as compared to controls (Fig. S3D-E). Taken together, these data confirm previous findings¹⁰ and show that, although cells are able to complete mitosis with damaged DNA, this leads to increased chromosome segregation defects.

Mitotic damage affects TPX2 protein level and Aurora A/TPX2 complex formation

Binding of TPX2 exerts a double effect on AurA: it stretches its activation segment in a way that makes AurA competent to bind substrates and it additionally protects the T-loop site T₂₈₈²² from PP1-dependent dephosphorylation.²⁸ Considering this, we asked whether the observed IR-induced loss of phosphorylation at T₂₈₈ (Fig. 1B) was dependent upon the release of TPX2 and consequent deprotection of the AurA T-loop site. To this end, we first examined whether PP1 is activated by mitotic DNA damage, monitoring the extent of phosphorylation at the inhibitory site T₃₂₀,²⁹ dephosphorylation at this site is closely associated with activation of PP1. In synchronized cells, CDK1-dependent phosphorylation of T₃₂₀ was maximal at 10h post-release (Fig. S4). Irradiation of mitotic cells caused an almost complete dephosphorylation of T₃₂₀ within 1h, indicative of PP1 activation, which coincided with a rapid decrease in TPX2 levels (Fig. 3A). On the other hand, CycB1 appeared to be degraded less efficiently in irradiated cells as compared to control cells (Fig. 3A). A similar pattern of PP1 activation was observed in double-thymidine synchronized cells treated with nocodazole at 7h and irradiated at 10h post-release (Fig. 3b). In this setting and relative to control cells, the level of TPX2 protein was clearly decreased 1h after administration of IR, with the effect more pronounced at subsequent time points; the level of AurA and Cyclin B1 remained unchanged at these later times (Fig. 3B). In agreement with the finding that a lower amount of TPX2 was present upon

DNA damage (Fig. 3B, 12h IR), immunoprecipitation of AurA from nocodazole-arrested cells showed that the amount of AurA-bound TPX2 was decreased upon irradiation (Fig. 3C, 12h IR).

Effect of mitotic damage on TPX2 protein stability

Considering that TPX2 is normally degraded at mitotic exit by APC/C in a CDH1-dependent manner³⁰ and that DNA damage causes premature activation of CDH1 in G2,³¹ we asked whether the rapid decrease in TPX2 level that we observed upon irradiation of mitotic cells reflected an untimely activation of CDH1. RNAi-mediated CDH1 downregulation (Fig. 4A) led to an evident rescue of TPX2 protein levels in irradiated and control cells (Fig. 4B). This was also the case for another CDH1 target, namely AurA (Fig. S5). To substantiate these data and confirm the ubiquitination-dependent degradation of TPX2, synchronized cells were irradiated in mitosis and treated with the proteasome inhibitor MG-132 15 min after irradiation. Analysis of cells extracts by SDS-PAGE showed that the presence of MG-132 led to a significant rescue of TPX2 levels (Fig. 4C).

Among the possible reasons for rapid changes in protein level is an increase in the rate of proteolytic degradation. To assess whether this was the case for TPX2, we determined its half-life ($T_{1/2}$) in control and IR-treated mitotic cells. The data showed an accelerated degradation of TPX2, which was only evident at early time points (Fig. 5A-B).

Taken together, this data show that, although premature activation of the APC/C-CDH1 by irradiation may affect the initial rate of TPX2 degradation, effects on protein stability are likely not sufficient to fully explain the effect of IR on steady state levels of TPX2 protein.

TPX2 expression is not affected by mitotic DNA damage

The observations above led us to ask whether transcriptional effects may have a role in the response of TPX2 to IR. To this end, we quantified TPX2 mRNA by real-time quantitative PCR. Analysis of samples showed no difference (11h) or a small decrease (12h) in TPX2 mRNA levels in IR treated cells as compared to control cells (Fig. 6A); such small effects at 12h following IR were not observed for AurA mRNA (Fig. 6B). In contrast, the level of CycB1 mRNA appeared to be increased by DNA damage

at both time points examined (Fig. 6C). The transcriptional response to IR displayed by AurA and CycB1 closely matched the pattern of protein expression observed by Western blotting (Fig. 3A). In contrast, the large difference between the pattern of TPX2 mRNA and protein expression and steady state protein levels suggested a post-transcriptional mode of regulation in this case.

Since IR did not apparently affect the stability of TPX2 mRNA (Fig. 6A), we analyzed the proportion of TPX2 mRNA actively associated with polysomes and hence being used for translation. Upon fractionation of ribosomes on sucrose gradients, we observed that TPX2 mRNA was less efficiently recruited to polysomes in DNA-damaged cells as compared to non-irradiated controls (Fig. 6D). This indicates that the translation of TPX2 mRNA is likely hindered upon irradiation of mitotic cells.

DISCUSSION

DNA damage-induced arrest at the G2/M transition of the cell cycle is contributed by inhibition of CDK1 as well as other mitotic kinases.³² We have previously shown that AurA, which controls ploidy by ensuring spindle bi-polarity,³³ is inhibited in response to the generation of double strand breaks in G2.¹⁶

In the present study we addressed the molecular mechanism by which DNA damage response pathways restrain AurA activity. To this end, we decided to examine cells transiting through mitosis, the time of maximal AurA activation. To correctly interpret the cellular response observed, we first addressed how genotoxic stress affects transition through mitosis. Separate lines of evidence derived from flow cytometry, analysis of phosphorylation of the CDK1 inhibitory site Y₁₅ and, particularly, time-course visual scoring of cells, demonstrated that the molecular events examined in our study were not attributable to a DNA damage-induced reversion to a G2-like state. The data showed that irradiated mitotic cells were able to transit through mitosis, in accordance with previous studies.¹⁰ These cells, however, showed significantly higher chromosome segregation defects followed by the formation of micronuclei.

In cells synchronized with nocodazole, which allowed maintaining constant levels of AurA during the course of the experiment, we observed a DNA damage-dependent inhibition of AurA activity, as visualized by dephosphorylation of the activating T-loop site T₂₈₈.^{25,26} In most kinases, phosphorylation of the T-loop site is of key importance for supporting enzymatic activity.^{32,34} The concomitant rapid activation of PP1 observed in irradiated cells led us to hypothesize an involvement of TPX2 in the mechanism of AurA inhibition by DNA damage. This reasoning was based on published evidence according to which physical interaction between AurA and TPX2 not only facilitates activation of the kinase²² but additionally protects the T-loop site T₂₈₈ of activated AurA from dephosphorylation by PP1.²⁸ In support to our hypothesis, we could demonstrate that dephosphorylation of T₂₈₈ in irradiated cells correlated with a decreased amount of TPX2 detectable in AurA immunocomplexes. Western blot analysis carried out at time points subsequent to irradiation revealed that the overall level of TPX2 was lower in irradiated than in control cells. This was particularly

evident upon nocodazole treatment, thus evidencing the net effect of DNA damage over pathways normally activated at mitosis.

We found that the fast degradation of TPX2 in response to IR occurred in an APC/C-CDH1-dependent manner. Published evidence indicates that DNA damage in the G2 phase of the cell cycle leads to premature APC/C-CDH1 activation.³¹ Our observations on the degradation of TPX2 show that also mitotic DNA damage may lead to unscheduled activation of CDH1. The fact that AurA was not degraded under these conditions may be explained by the finding that degradation of CDH1 targets occurs in a sequential manner depending on their extent of ubiquitination.³⁵ Premature activation of CDH1 is also supported by the decreased rate of CycB1 degradation that we observed upon irradiation, an event that is likely the consequence of CDH1-dependent inactivation of CDC20, in addition to IR-dependent increase of CycB1 mRNA level. The amount of CycB1 remaining upon irradiation did not, however, hinder the completion of mitosis by causing reversion to a G2-like state. Indeed, it was recently shown that, although the proteolysis of CycB1 is necessary for mitotic exit,³⁶ graded levels of mitotic cyclins delay, but do not block, mitotic exit in a dose-dependent manner.³⁷

A careful evaluation of the rate of TPX2 degradation revealed an initial rapid decrease of the protein's half-life in response to damage. We reasoned, however, that this could not entirely account for the effect of IR on the level of TPX2 in the cell. Upon examination of possible effects of DNA damage on mRNA, we observed that the overall level of TPX2 mRNA did not vary in response to ionizing radiation, indicating that changes in mRNA stability were also to be ruled out as explanation for the diminished overall level of TPX2 protein.

In addition to the mere stability of mRNA, other mechanisms contribute to control translation. It is known that in response to hormonal stimulation of oocyte maturation in *Xenopus*, translationally quiescent mRNAs coding for regulators of the meiotic division undergo first elongation of their short poly(A) tails and, subsequently, translation.³⁸ In general, poly(A) tail elongation facilitates translation, whereas shortening or removal of poly(A) tails correlate with silencing.³⁹ It is also established that ribosomes are tightly associated with microtubules and that localized translation of a conserved group of mRNAs enriched on microtubules plays an important role at the onset of mitosis.⁴⁰ Among these is the mRNA coding for TPX2.⁴¹ Considering that

poly(A) tail elongation in *Xenopus*⁴² and mammalian cells⁴³ was shown to depend on phosphorylation events catalyzed by AurA, it is conceivable that the DNA damage-dependent inhibition of AurA activity observed in our study may result in decreased polyadenylation of mRNAs coding for mitotic regulators. Interestingly and in support to this reasoning, we found that DNA damage hampered the recruitment of TPX2 mRNA on polysomes, the actively translating population of ribosomes. Poly(A) tail-binding protein (PABP) along with its interacting partner eIF4G has been reported to displace the inhibitory protein eIF4E-BP1 from eIF4E, and in such manner favor cap-dependent mRNA translation.⁴⁴ Based on our data we hypothesize that decreased polyadenylation may be the cause of reduced recruitment of TPX2 mRNA on polysomes. Further work will be required to address this possibility.

Along this line of speculation, it is conceivable that CDK1 may also play a role in the control of TPX2 mRNA translation. High CDK1 activity was reported to promote recruitment to polysomes of mRNAs coding for mitotic regulators, at least in sea urchins.⁴⁵ Whether also the recruitment of TPX2 mRNA is controlled in this manner was not assessed by the authors, though the DNA damage-dependent inhibition of CDK1 activity in mitosis,¹² indirectly observed in our study by activation of PP1, would support such possibility. On the other hand, the previously reported inhibition of general translation mediated by phosphorylation of eIF2-alpha upon UV damage,⁴⁶ a mechanism that was recently claimed to reduce Cdc25B mRNA translation,⁴⁷ cannot be invoked to explain our results. We observed a selective effect of IR on the translation of TPX2 mRNA and the resulting protein but not a general inhibition of translation, as evidenced by unvaried protein level of other mitotic players such as AurA or CycB1.

In conclusion, this study exposes the molecular mechanism that controls AurA in response to genotoxic damage. The data show that irradiation of cells during mitosis leads to inhibition of AurA activity through failed association with its partner TPX2, leading to dephosphorylation of the AurA T-loop site T₂₈₈. Degradation of TPX2 through APC/C-CDH1 ubiquitination partially contributes to reduce TPX2 protein level in the initial phases of the response to DNA damage. Additionally, we observed a selective inhibition of TPX2 mRNA loading on polysomes in response to IR. Considering that each ribosome on the polysome is actively engaged in producing

TPX2 polypeptide, the reduced loading of TPX2 mRNA on polysomes, along with the rapid protein degradation, probably explains the effects of IR on steady state levels of TPX2 protein. In turn, failed replenishment of the rapidly turning-over TPX2 pool leads to PP1 dependent dephosphorylation and inactivation of AurA.

MATERIALS AND METHODS

Antibodies and chemicals

The following antibodies were used: AurA (Pab 35, purified rabbit polyclonal; 35C1, mouse monoclonal¹⁶); AurA-pT₂₈₈ (Biosource; rabbit polyclonal); TPX2 (GenWay, mouse monoclonal); PP1 (Santa Cruz Biotech., mouse monoclonal); PP1-pT₃₂₀ (Cell Signaling Tech., rabbit polyclonal); H3-pS₁₀ (Millipore; rabbit polyclonal); Cyclin B1 (Upstate Biotech. Inc., mouse monoclonal); MSH2 (Santa Cruz Biotech., rabbit polyclonal sc-494); CDK1-pY₁₅ (Cell Signaling Tech., rabbit polyclonal); CDH1 (Santa Cruz Biotech., mouse monoclonal sc-56312); CHK2-pT₆₈ (Santa Cruz Biotech., rabbit polyclonal sc-16277-R); HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (GE-Healthcare); Alexa Flour-488 and -594 conjugated secondary antibodies (Invitrogen).

Thymidine (SynGen Inc.) was dissolved in PBS and filter-sterilized. Nocodazole (Sigma) and MG132 (Calbiochem) were dissolved in DMSO and stored in aliquots at -20 °C. Cycloheximide (CHX) (Calbiochem) was dissolved in ethanol and stored at -20 °C.

Cell culture and siRNA treatment

HeLa cells were maintained and synchronized by a double-thymidine block (DTB) release as described.¹⁶ For RNAi experiments, cells were seeded in 6-cm dishes and immediately transfected with 10 nM CDH1 siRNA (Sigma) using the Lipofectamin RNAiMAX reagent (Invitrogen) for 24h, followed by synchronization with DTB.

Flow cytometry

Cell cycle analysis by flow cytometry was performed upon propidium iodide (PI, Molecular Probes) staining of DNA as described.¹⁶ Cells were examined using a Dako CyAnTM ADP instrument and Summit software.

Immunofluorescence

Indirect immunofluorescence was performed with cells grown on glass cover slips. At indicated time points, cover slips were rinsed in PBS, fixed in 100% ice-cold methanol and stored at -20°C. Cover slips were blocked with 3% milk-PBS and gently shaken (15-20 min, RT). Overnight incubation with primary antibodies was

carried out in a wet chamber at 4°C. The next day, cover slips were washed twice in 3% milk-PBS (10 min) with gentle shaking and probed with secondary antibodies (1h, RT in the dark). Cover slips were finally washed twice with PBS (10 min), rinsed in ddH₂O and mounted on Vecta-shield DAPI (Vector Labs) solution. Cells were observed with a Leica DMRB microscope equipped with a 100W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (HCX PL APO 63X) and images were captured with a Leica DFC 360 FX camera. Images were obtained using the Leica Application Suite[®] software.

Calculation of mitotic index

Synchronized HeLa cells stained with DAPI and H3-pS₁₀ polyclonal antibody were imaged with a Leica DMRB microscope as described above. At least 300 cells were counted for each time point from two independent experiments. H3-pS₁₀ positive cells were considered as mitotic. Standard deviation was determined from the percent values of the two sets.

Time-Lapse Microscopy

HeLa cells were grown on 3.5-cm glass-bottom dishes (Mat Tek). Cells were imaged on an Olympus IX81 fluorescence microscope equipped with an external temperature control chamber and CO₂ cylinder set to maintain the cells at 37°C with 5% CO₂. Images were captured every 2 min using a CCD camera (Orca AG, Hamamatsu) using the cellR[®] software (Olympus).

Western blotting, immunoprecipitation and kinase assay

Whole-cell extracts (WCE) were prepared using a modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 0.1% SDS, 0.5 mM Na-orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) or immunoprecipitation (IP) buffer.¹⁶ For IP cells were lysed and proteins resolved as described.¹⁶ For kinase activity assay, immunoprecipitated proteins were additionally washed with 2x 1ml NEB3 buffer (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl and 1 mM DTT) and assays performed as described.²⁵

Polysome profiling and RT-PCR

Cell pellets from a synchronized population of HeLa cells treated in the presence or the absence of IR were prepared as described.⁴⁸ Total RNA was isolated from cell pellets using the GenElute™ Mammalian Total RNA Kit (Sigma) and quantified using a spectrophotometer. RNA from the 80S or the polysome fractions was extracted with TRIzol-LS (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III reverse transcription (Invitrogen) from 1 µg RNA. Target genes expression levels were determined by qPCR using a Fast EvaGreen master mix (Biotium) and with the following primer pairs:

TPX2 Fwd: 5'-ATAGATTCATGGTTTGAGGAGAAG-3'

TPX2 Rev: 5'-CTTTGTAGTAAGTGTTGTCAACTG-3'

AurA Fwd: 5'-TCCAGGAGGACCACTCTCTG-3'

AurA Rev: 5'-GGGAATGTGAATTCAACCCGTG-3'

CycB1 Fwd: 5'-GAATGGACACCAACTCTACAAC-3'

CycB1 Rev: 5'-TGACAGTCATGTGCTTTGTAAGTC-3'

The house-keeping gene porphobilinogen deaminase (PBGD) was used as control and amplified with the following primers:

Fwd: 5'-CAACGGCGGAAGAAAACAG-3'

Rev: 5'-TCTCTCCAATCTTAGAGAGTG-3'.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1 - Inhibition of AurA kinase activity in response to mitotic damage.

(A) The expression of selected mitotic proteins in DTB-released HeLa cells was analyzed at the indicated time-points after release. AS: asynchronous. PP1 was used as loading control.

(B) Western blot analysis of total (AurA) and active (AurA-pT₂₈₈) AurA protein kinase in cells synchronized by the DTB-release method. CHK2-pT₆₈ was used as read-out for the DNA damage response.

Figure 2 - Irradiation of mitotic cells does not impair the completion of mitosis.

(A) DTB-synchronized HeLa cells were irradiated (9 Gy) at 10h post-release and analyzed by Western blot at subsequent time-points for a mitotic marker (H3-pS₁₀) and DNA damage response (CHK2-pT₆₈). MSH2 was used as loading control.

(B) DTB-synchronized HeLa cells left untreated or irradiated (9 Gy, 10h) were analyzed by immunofluorescence for dephosphorylation of Y₁₅ in CDK1 as marker of mitotic entry. At least 200 events from 3 different fields per time-point were scored.

(C) Quantification of early (pro- and metaphase) and late (ana- and telophase) mitotic events in control and irradiated cells. Three different fields from two independent experiments were counted for each time-point.

Figure 3 - Effect of mitotic DNA damage on TPX2 protein level and AurA/TPX2 complex formation.

(A) DTB-synchronized HeLa cells were irradiated (9 Gy) at 10h post-release and analyzed by Western blot at subsequent time-points for PP1 activation (dephosphorylation of T₃₂₀), the expression of mitotic markers (TPX2, AurA and CycB1) and the DNA damage response (CHK2-pT₆₈). MSH2 was used as loading control.

(B) DTB-synchronized HeLa cells were treated with nocodazole (200ng/ml) at 7h and irradiated (9 Gy) at 10h post-release. PP1 activation as well as the expression of mitotic markers (CycB1, AurA and H3-pS₁₀), the level of TPX2 and the activation of the DNA damage response (CHK2-pT₆₈) were analyzed by Western blot.

(C) AurA was immunoprecipitated from WCEs shown in b) using a purified polyclonal antibody. AurA and its partner TPX2 were revealed with specific monoclonal antibodies. IgG(H), stained with Ponceau Red, was used as loading control.

Figure 4 - Mitotic damage affects TPX2 protein stability.

(A) HeLa cells were transfected with a specific siRNA to CDH1 for 24h, DTB-synchronized and either left untreated or irradiated (9 Gy) at 10h post-release. Expression of CDH1 was examined at the indicated time points. MSH2 was used as loading control.

(B) Cells treated as in a) were examined for the rescued expression of TPX2 as well as for activation of the DNA damage response (CHK2-pT₆₈) following irradiation. PP1 was used as loading control.

(C) DTB-synchronized HeLa cells were irradiated (9 Gy) at 10h post-release and either left untreated or treated with MG-132 (10 mM) 15 min post-IR. The rescued expression of TPX2 and the activation of the DNA damage response (CHK2-pT₆₈) were examined by Western blot. PP1 was used as loading control.

Figure 5 - Effect of mitotic damage on TPX2 protein half-life.

(A) CHX (10 mg/ml) was added to DTB-synchronized HeLa cells at the time of irradiation (9 Gy, 10h). The expression of TPX2 and the activation of the DNA damage response (CHK2-pT₆₈) were examined at the indicated time-points. PP1 was used as loading control. One experiment representative of three independent determinations is shown.

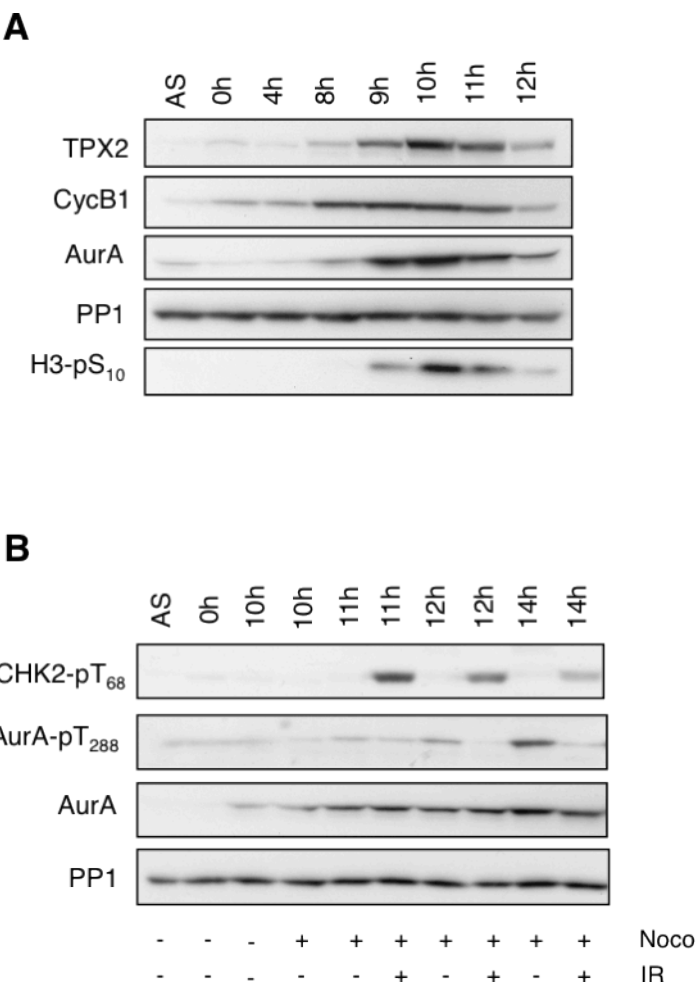
(B) TPX2 half-life was determined from quantification of the time-points shown in a) using ImageJ Analysis Software and PP1 as loading control.

Figure 6 - Effect of damage on mRNAs encoding mitotic proteins and on the recruitment of TPX2 mRNA to polysomes.

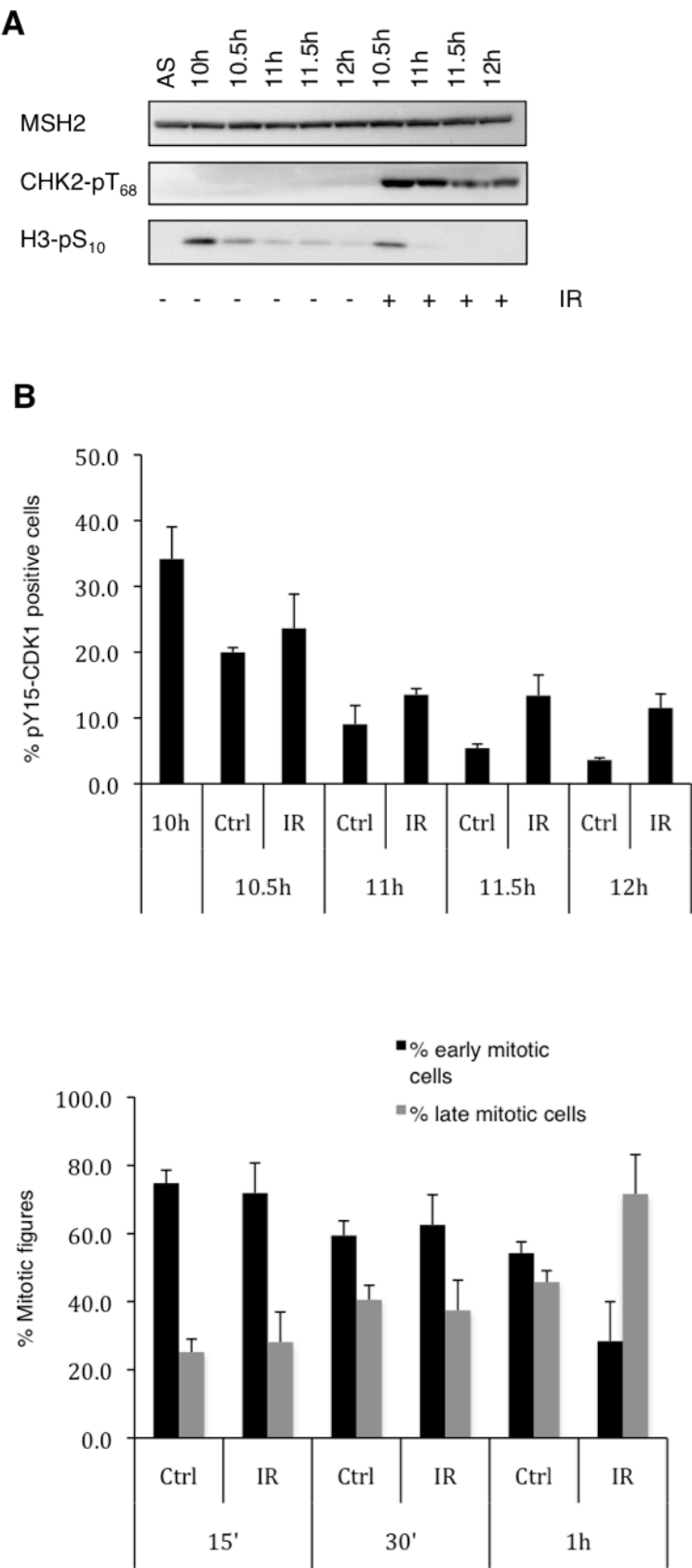
(A-C) Real-time PCR was performed at the indicated time points for quantification of TPX2, AurA and CycB1 mRNA obtained from control cells or cells treated with IR.

(D) Real-time PCR was performed at the indicated time point to quantify the TPX2 mRNA associated with the polysomal fraction (B) of a sucrose gradient. Data from

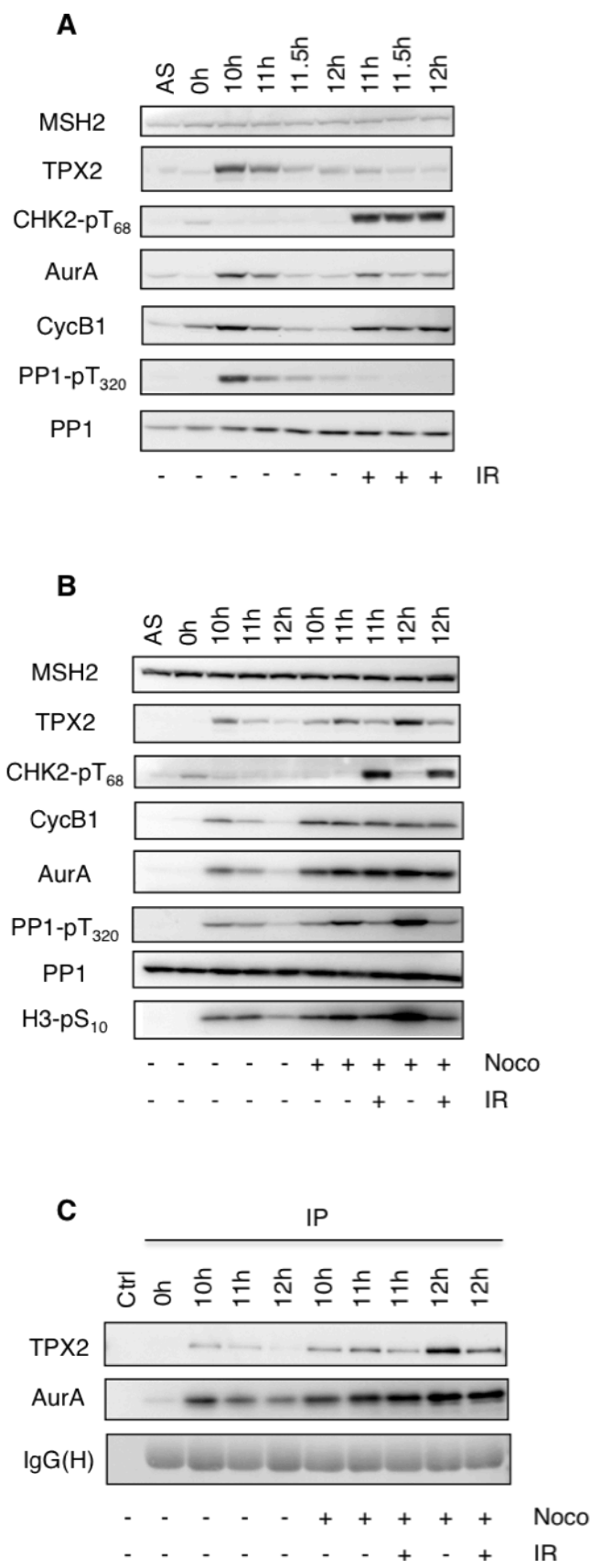
one of two independent experiments are shown. The fold-change over the TPX2 mRNA associated with the 80S ribosome fraction (set to 1) is shown.



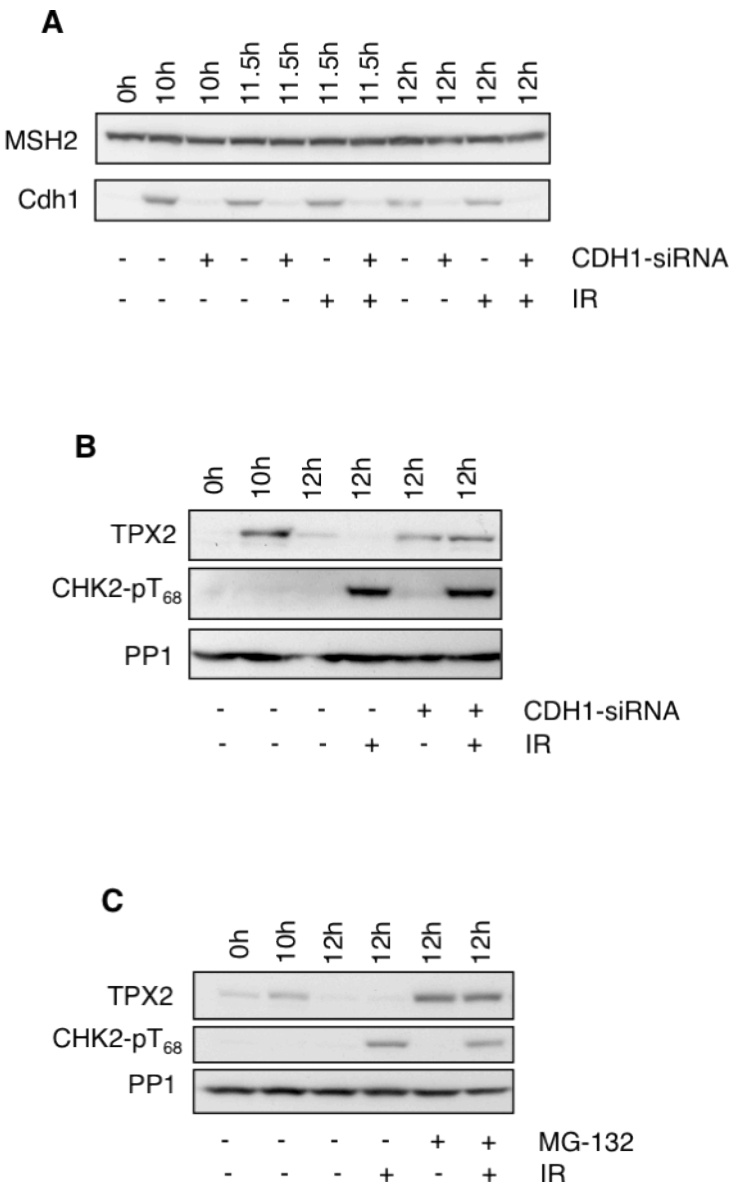
Bhatia et al., Figure 1



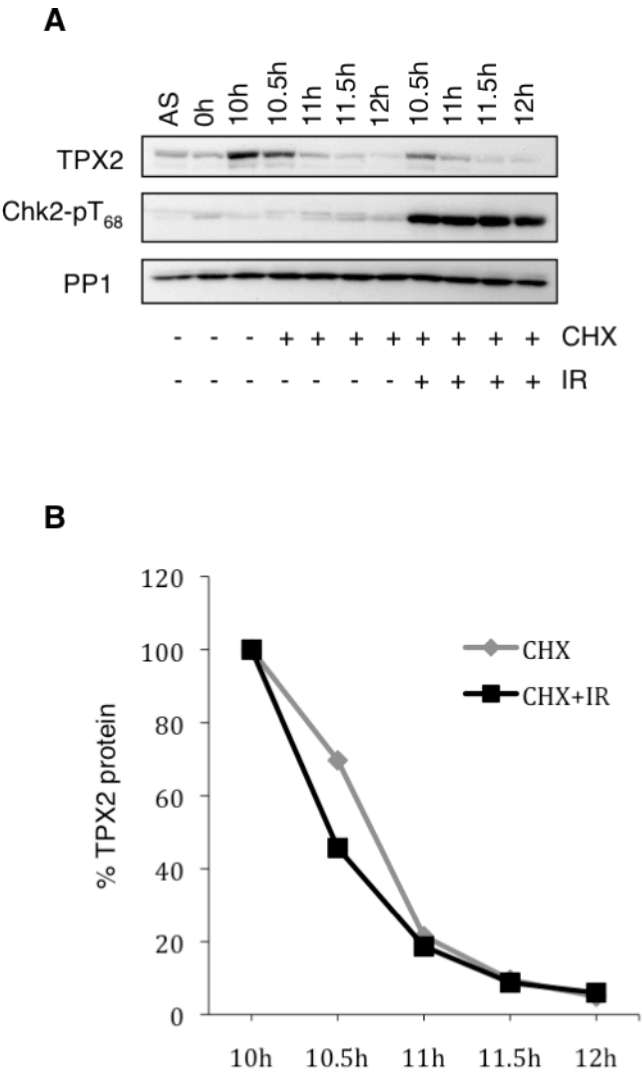
Bhatia et al., Figure 2



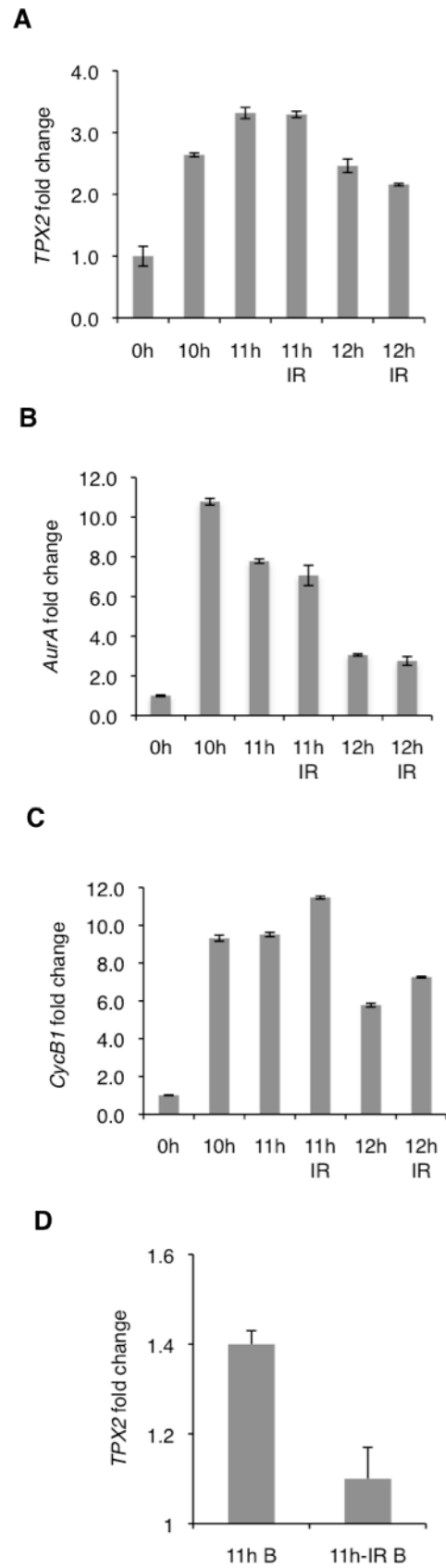
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Bhatia et al., Figure 4



Bhatia et al., Figure 5



Bhatia et al., Figure 6

SUPPLEMENTARY MATERIAL TO:

Mitotic DNA damage targets the Aurora A/TPX2 complex

Payal Bhatia¹, Mirco Menigatti¹, Michele Brocard², Simon Morley² and Stefano Ferrari^{1,3}

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1 - HeLa cells synchronization and AurA kinase activity.

(A) Flow cytometric analysis of HeLa cells synchronized by the DTB-release protocol (see Material and Methods). DNA was stained with PI.

(B) DTB-synchronized HeLa cells were released and fixed at the indicated time points. Staining with the H3-pS₁₀ antibody was used to determine the mitotic index. At least 300 cells were counted for each time-point. Data from one of two representative experiments are shown. Green: H3-pS₁₀; blue: DAPI.

(C) Kinase activity assay for AurA immunoprecipitated from extracts of DTB-released HeLa cells.

(D) Flow cytometric analysis of HeLa cells synchronized by DTB-release, treated with nocodazole (200 ng/ml) at 7h and irradiated (9 Gy) at 10h post-release. DNA was stained with PI.

Supplementary Figure S2 - Effect of IR on the transition through mitosis.

(A) Flow cytometric analysis of HeLa cells synchronized by DTB and irradiated (9 Gy) at 10h post-release. DNA was stained with PI.

(B) CDK1 activation at mitosis in cells treated as in a) was determined by the pattern of dephosphorylation of the inhibitory site Y₁₅. At least 200 cells were counted for each time-point.

(C) A representative field of the events plotted in b) is shown. Green: CDK1-pY₁₅; blue: DAPI.

(D) Live cell imaging of HeLa cells synchronized by DTB and irradiated (9 Gy) at 10h post-release. Transition through mitosis was imaged for a period of 1h in cells examined at the time of irradiation (0h) or 1h upon irradiation (1h).

Supplementary Figure S3 - Chromosome alignment and segregation defects caused by irradiation.

(A) Misaligned (arrowheads) or lagging chromosomes (arrows) were scored in metaphases from control cells or cells treated with IR (9 Gy). At least 100 metaphases were counted from two independent experiments. DNA was stained with DAPI.

(B-C) Statistical analysis of the events visualized in a).

(D) Micronuclei (arrows) formed upon mitotic exit in control or IR-treated cells. At least 200 events were counted from two independent experiments. DNA was stained with DAPI.

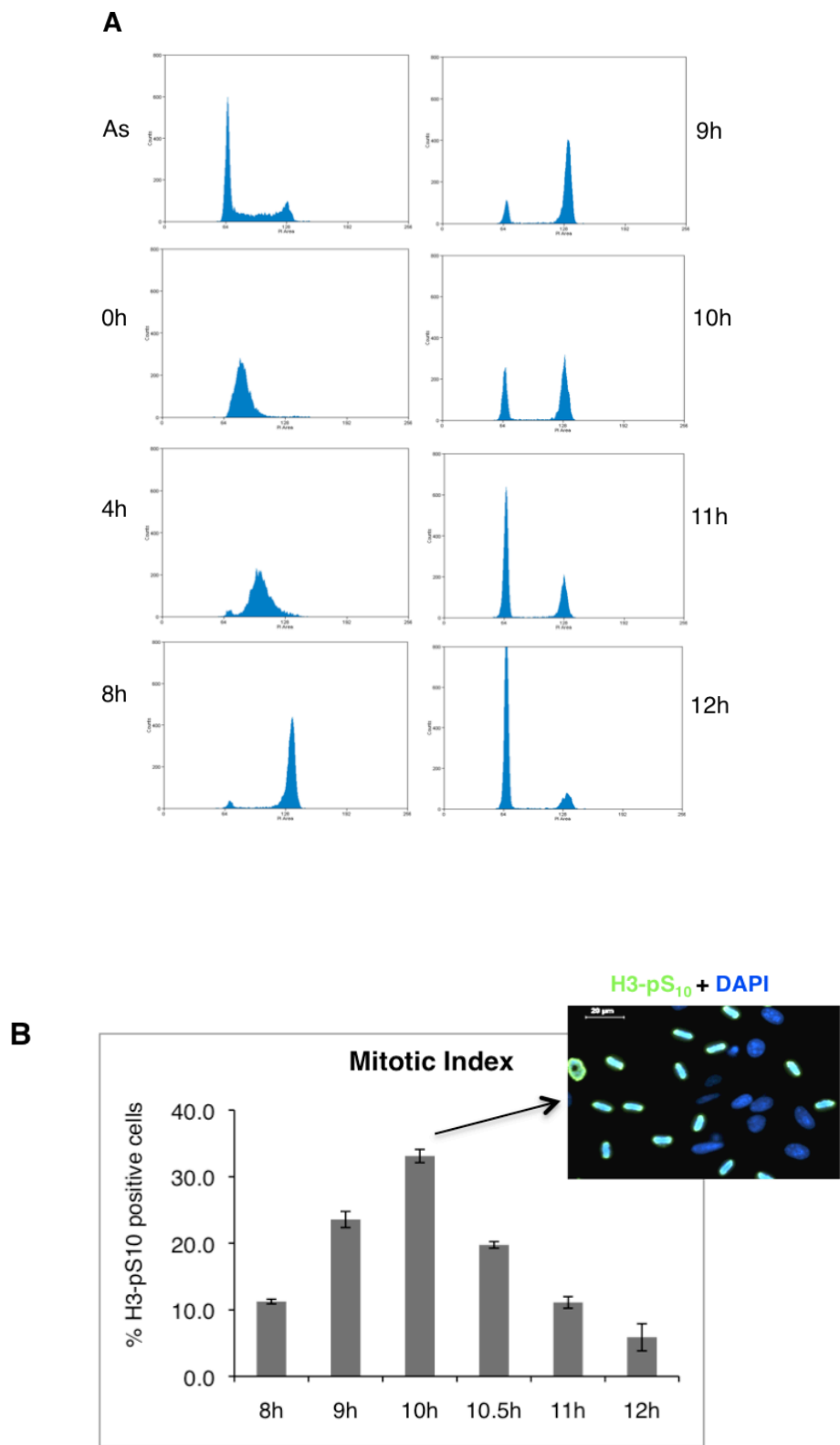
(E) Statistical analysis of the events visualized in d).

Supplementary Figure S4 – Mitotic inactivation of PP1.

Mitotic phosphorylation at the site T₃₂₀ in PP1, indicative of inhibition of phosphatase activity, revealed by Western blot analysis of WCE obtained from DTB-released HeLa cells.

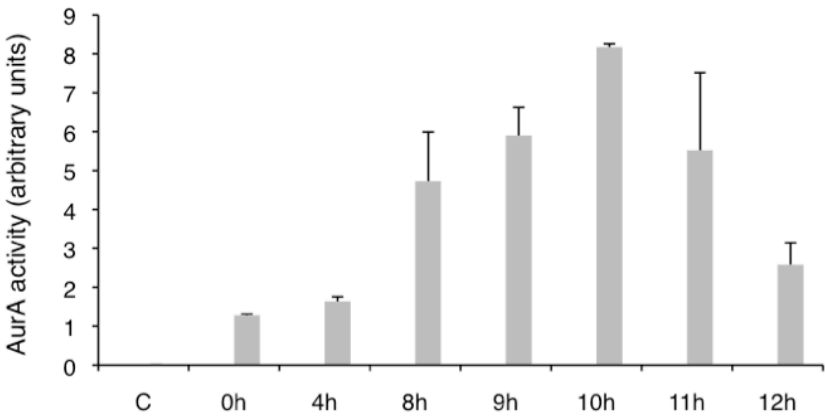
Supplementary Figure S5 – Effect of CDH1 downregulation on AurA protein stability.

HeLa cells were transfected with an siRNA specific to CDH1 for 24h, DTB-synchronized and either left untreated or irradiated (9 Gy) at 10h post-release. Expression of AurA was examined at the indicated time point. MSH2 was used as loading control.

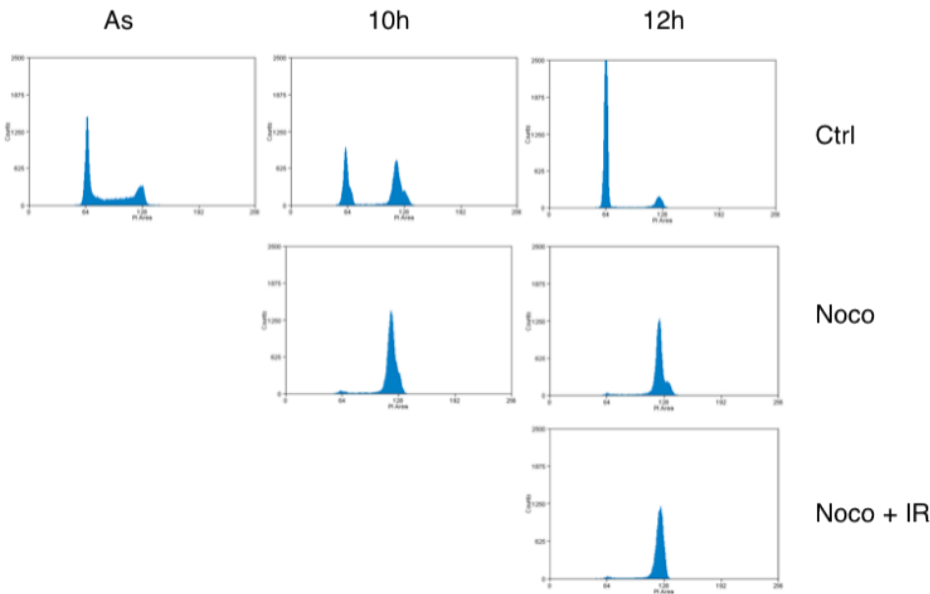


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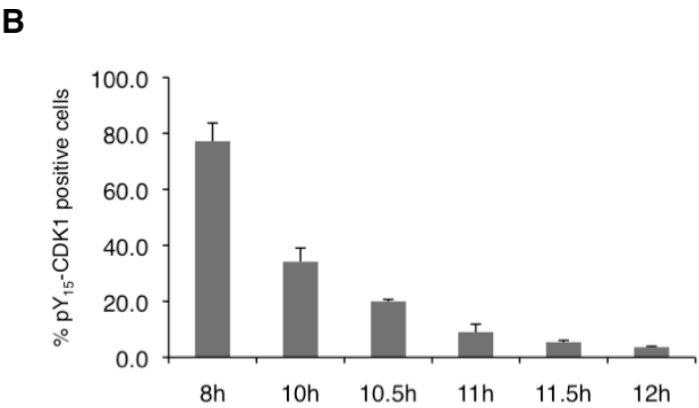
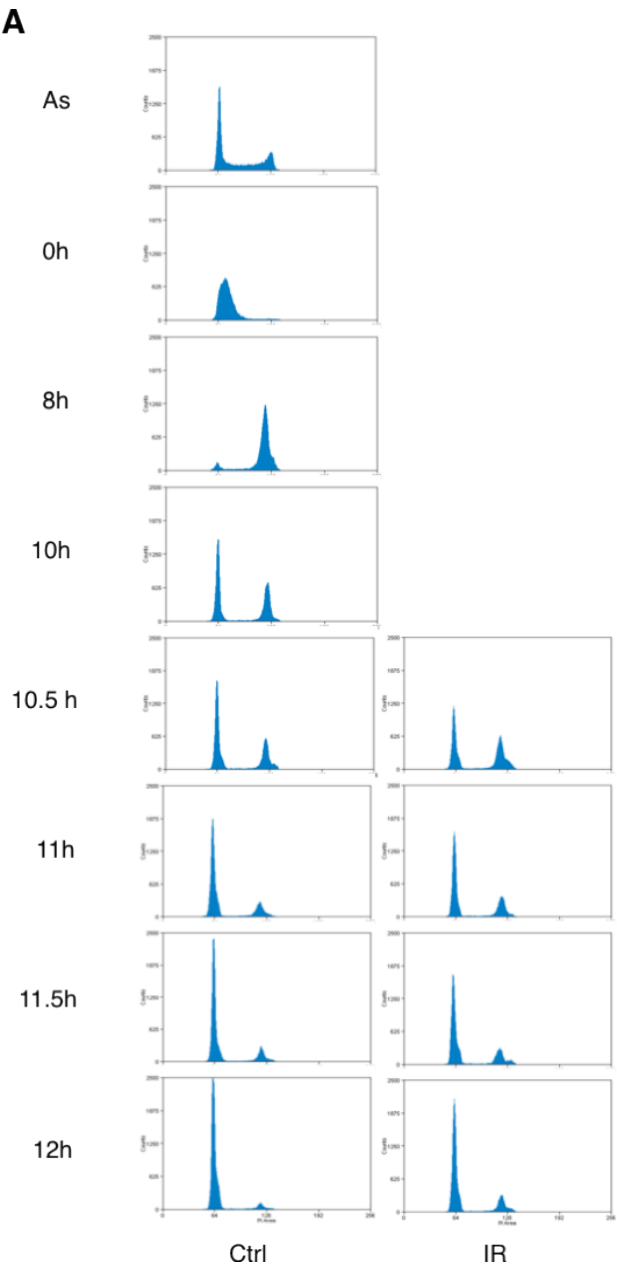
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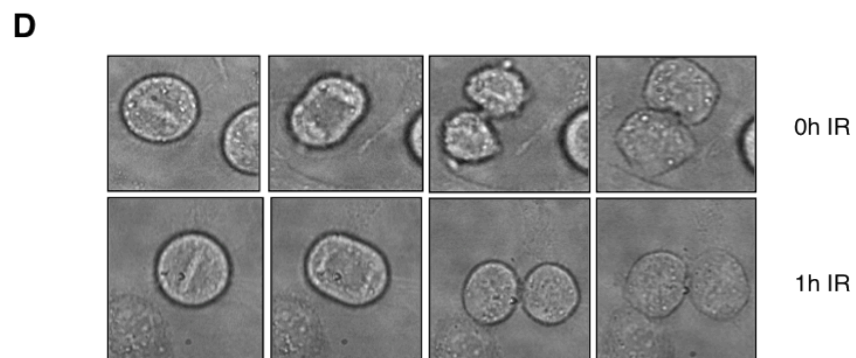
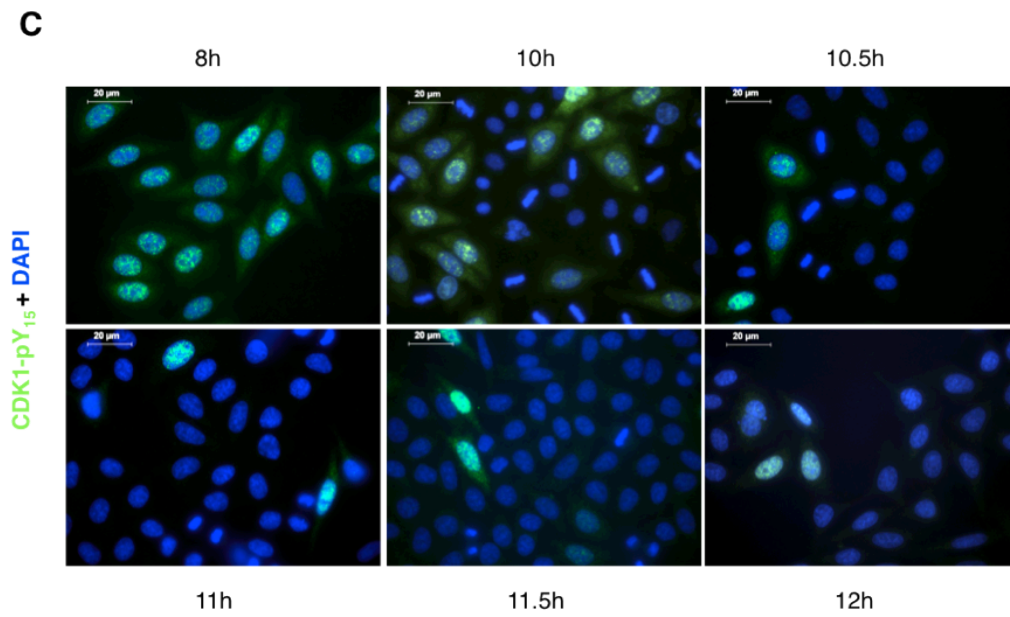
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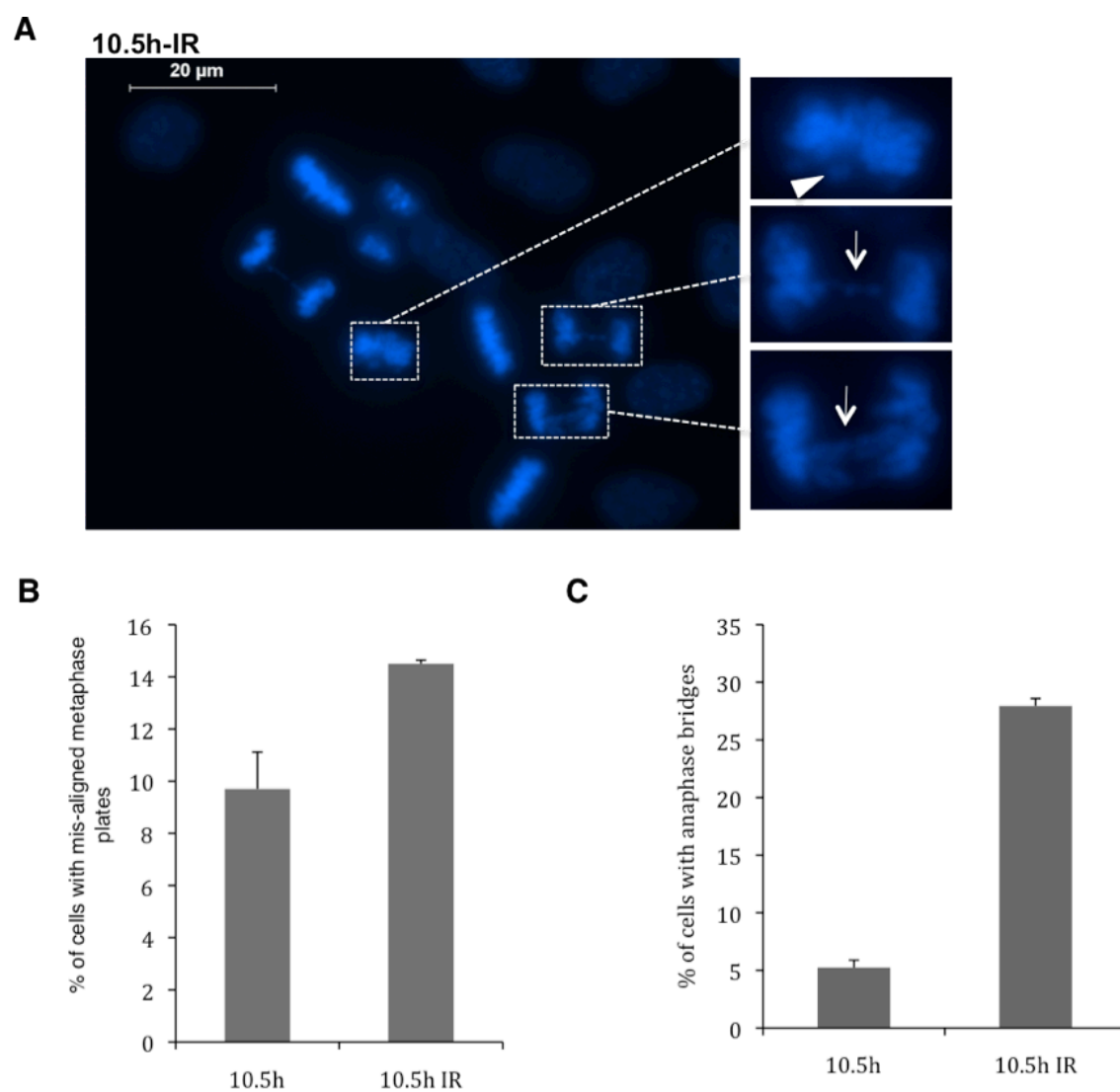
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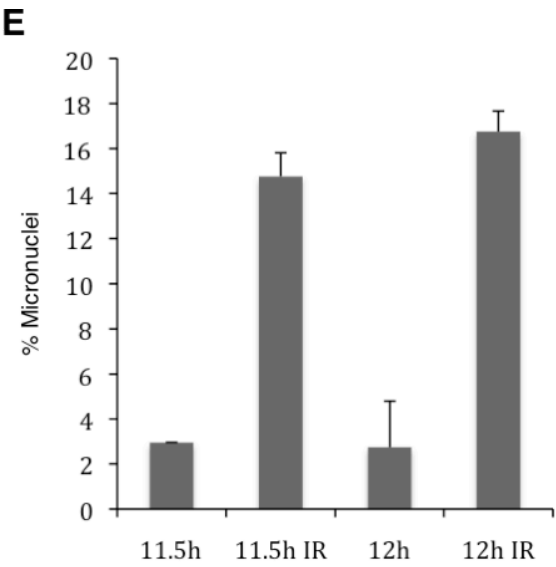
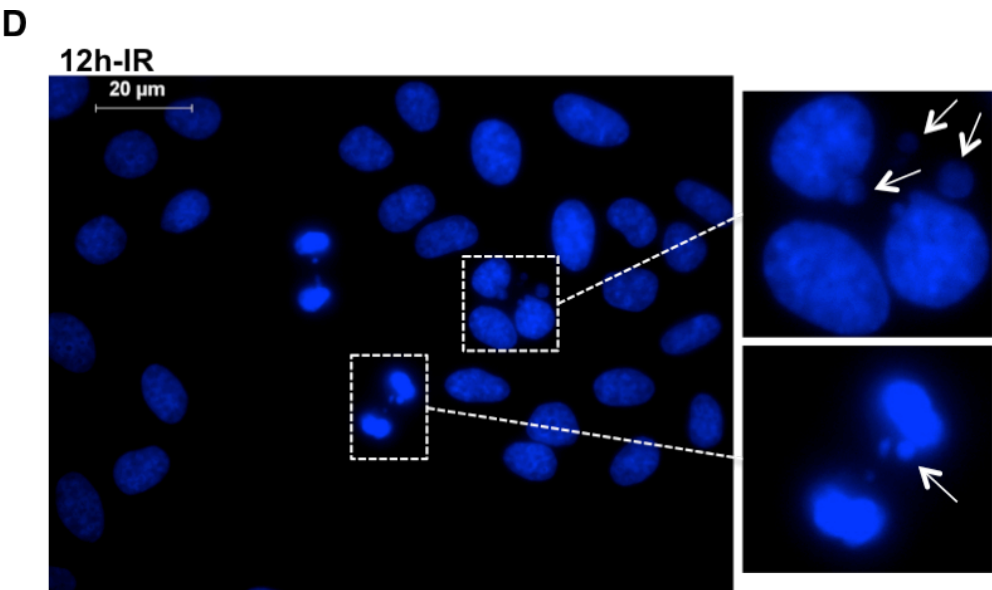
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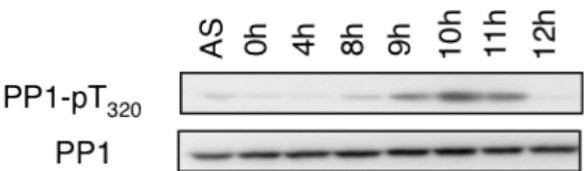
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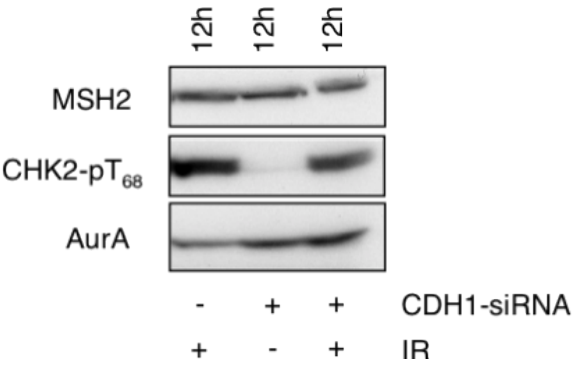
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PART 2- UNPUBLISHED DATA

Cell Cycle Progression Post DNA Damage Induced During Prometaphase

(Continuation of 1st part of the results presented above)

4.2 CELL CYCLE PROGRESSION POST DNA DAMAGE INDUCED DURING PROMETAPHASE

AIM

DNA damage induced in cells transiting through mitosis results in different cellular fates, such as cell cycle arrest or reversion to G2, depending upon the point at which damage is caused. Earlier reports have suggested that the cell cycle arrest observed in response to DNA damage in prometaphase is due to activation of spindle assembly checkpoint (SAC) (Choi and Lee, 2008), (Mikhailov et al., 2002). The aim of this part of my study was to clearly establish how cells respond to damage administered in prometaphase. As we observed that damage induced during metaphase does not impede cell cycle progression and that cells completed mitosis much like the control cells, although with marked increase in chromosomal abnormalities (see above), we decided to study the effect of damage during prometaphase to get a full picture of the DNA damage response in mitotic cells.

4.2.1. RESULTS

4.2.1.1. DNA damage induced in mitotic cells generates double-stranded breaks (DSBs)

In order to understand the effect of DNA damage on mitotic cells, we first examined whether damage induced by IR in synchronized mitotic cells resulted in the formation of DSBs. To this end, cells were synchronized with DTB followed by nocodazole and irradiated 2h before fixation for immunofluorescence. Nuclei were stained with γ -H2AX antibody, to score for the presence of DSBs and H3-pS₁₀ antibody, to identify mitotic cells. As shown in Fig.1, IR-induced damage resulted in the formation of DSBs (γ -H2AX foci) (Fig. 1B), which were absent in the non-irradiated control cells (Fig. 1A).

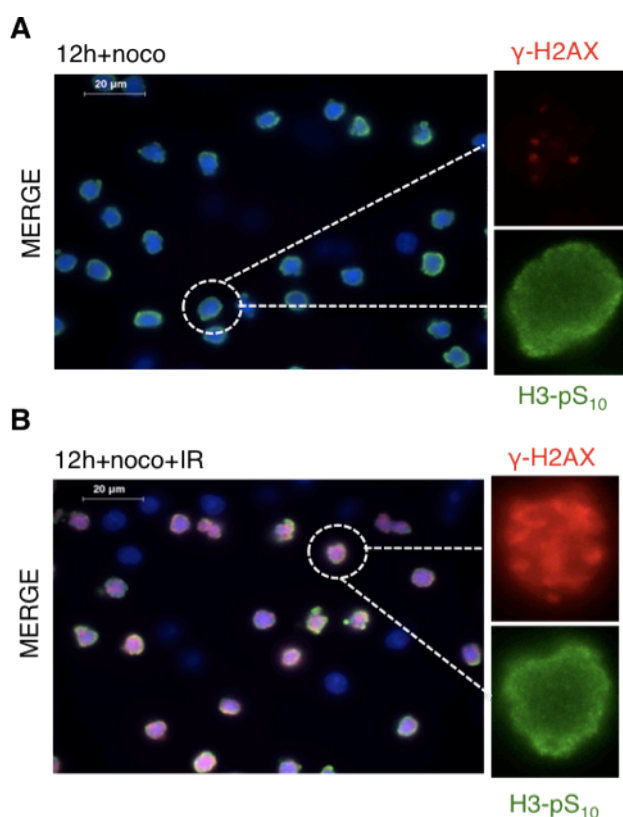


Figure 1- IR administered in mitosis results in DNA damage induced DSBs. HeLa cells were synchronized with DTB and nocodazole (7h, 200 ng/ml) and either A) left untreated or B) treated with 9 Gy IR at 10h and analyzed at 12h. Blue: DAPI, Red: γ -H2AX and Green: H3-pS₁₀.

4.2.1.2. DNA damage induced in nocodazole-arrested cells does not block cells in mitosis

Previous studies have established that damage induced in nocodazole-treated cells result in either mitotic arrest, due to activation of the spindle assembly checkpoint (SAC) (Choi and Lee, 2008), (Mikhailov et al., 2002) or a reversion to G2-like state (Chow et al., 2003), upon removal of nocodazole. In order to establish the fate of mitotic cells in our experiments, namely to determine whether or not cells remain blocked in mitosis after DNA damage, we performed Western blotting and flow-cytometry. Cells were synchronized with nocodazole and, immediately after releasing into fresh medium, damage was induced with IR (9 Gy) and analyzed at the indicated time points. As shown in Fig. 2A, probing with the mitotic marker H3-pS₁₀ showed that cells were not apparently blocked in mitosis, since H3-pS₁₀ phosphorylation disappeared with time, indicative of chromosome decondensation. DSBs induced upon administration of the topoisomerase-II inhibitor etoposide gave a similar result (Fig. 2B). In addition, as determined by flow-cytometry (Fig. 2C), we could confirm that cells progressed into the next cell cycle much like control cells, despite the presence of damage: should the cells have reverted to a G2-like state, the G2-M peak of damaged cells would have been higher as compared to the controls.

To clearly distinguish mitotic exit from reversion of cells to G2, we performed time-lapse imaging of cells. As shown in Fig. 2D, damaged cells appeared to exit mitosis without arresting or reverting to a G2-like state. These results indicated that DNA damage induced in mitosis does not cause cell cycle arrest and cells complete mitosis much like the controls.

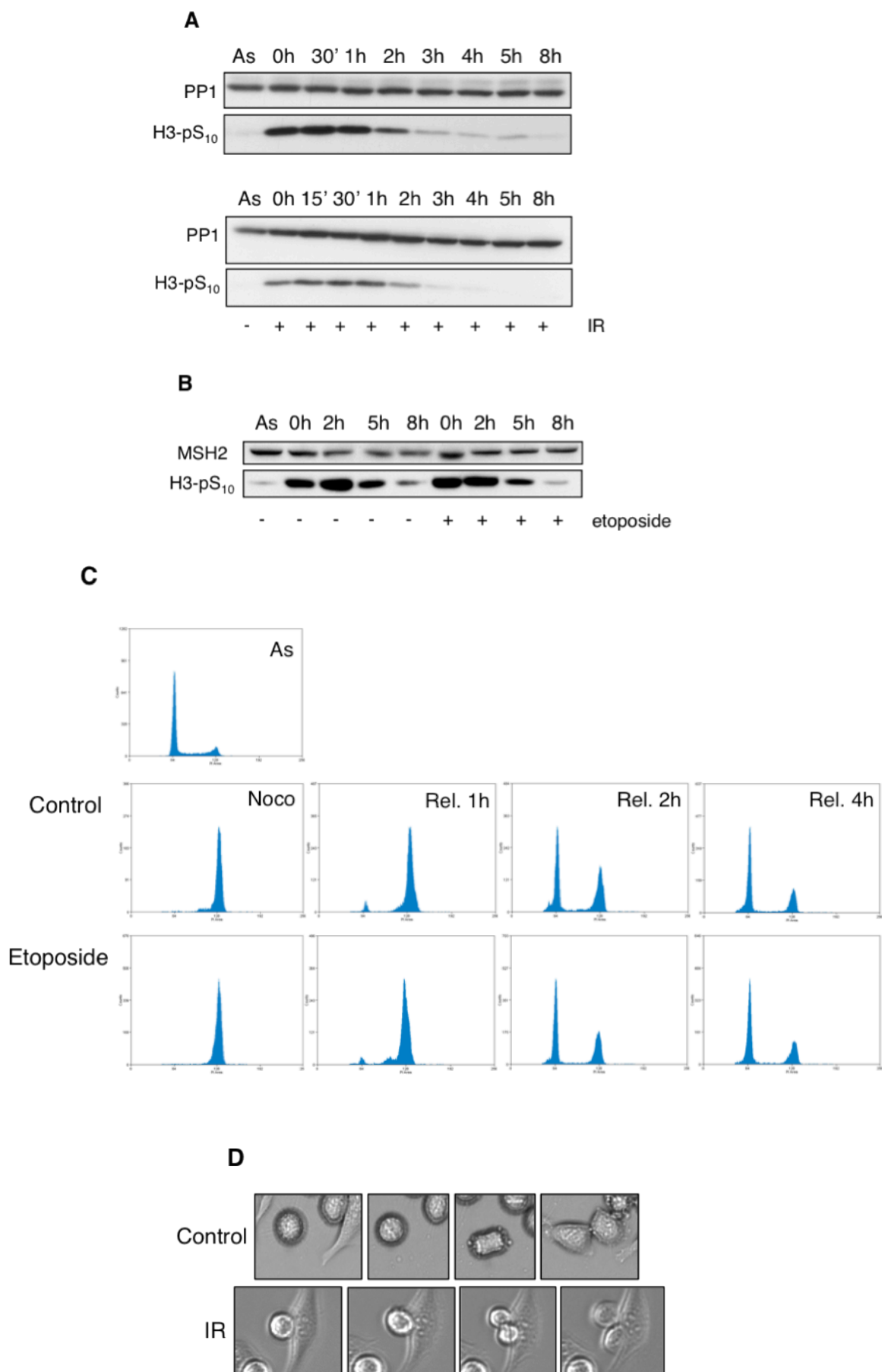


Figure 2- Irradiation of mitotic cells does not impair completion of mitosis. A) HeLa cells were treated with nocodazole (200 ng/ml) for 14h, released into fresh medium and immediately treated with IR (9 Gy) and collected at the indicated time points. B) HeLa cells were treated with nocodazole (400 ng/ml) for 15h followed by a 1h treatment with etoposide (5 μ M), released into fresh medium and collected at the indicated time points. C) Cells were treated as in b) and analyzed by flow-cytometry. DNA was stained with PI. D) Cells were synchronized by DTB and nocodazole (7h, 200 ng/ml), left untreated or treated with IR at 10h (9 Gy) for 2h and imaged every 5 min for 3h after release from nocodazole.

4.2.1.3. Cell cycle progression occurs with increased chromosomal segregation defects

As cells completed mitosis despite the presence of damage, we attempted to assess the status of the daughter cells that originated from a mitotic-irradiated mother cell. As shown in Fig. 3, synchronized and IR-treated HeLa cells stained with DAPI, H3-pS₁₀ and γ -H2AX showed the presence of chromosomal bridges and micronuclei. The DNA in the bridges appeared to be condensed chromatin, as seen by H3-pS₁₀ staining. The micronuclei, in contrast, had decondensed DNA but retained the damage-induced γ -H2AX foci. This indicated that mitosis proceeded despite the presence of unrepaired damaged structures and resulted in severe chromosomal abnormalities.

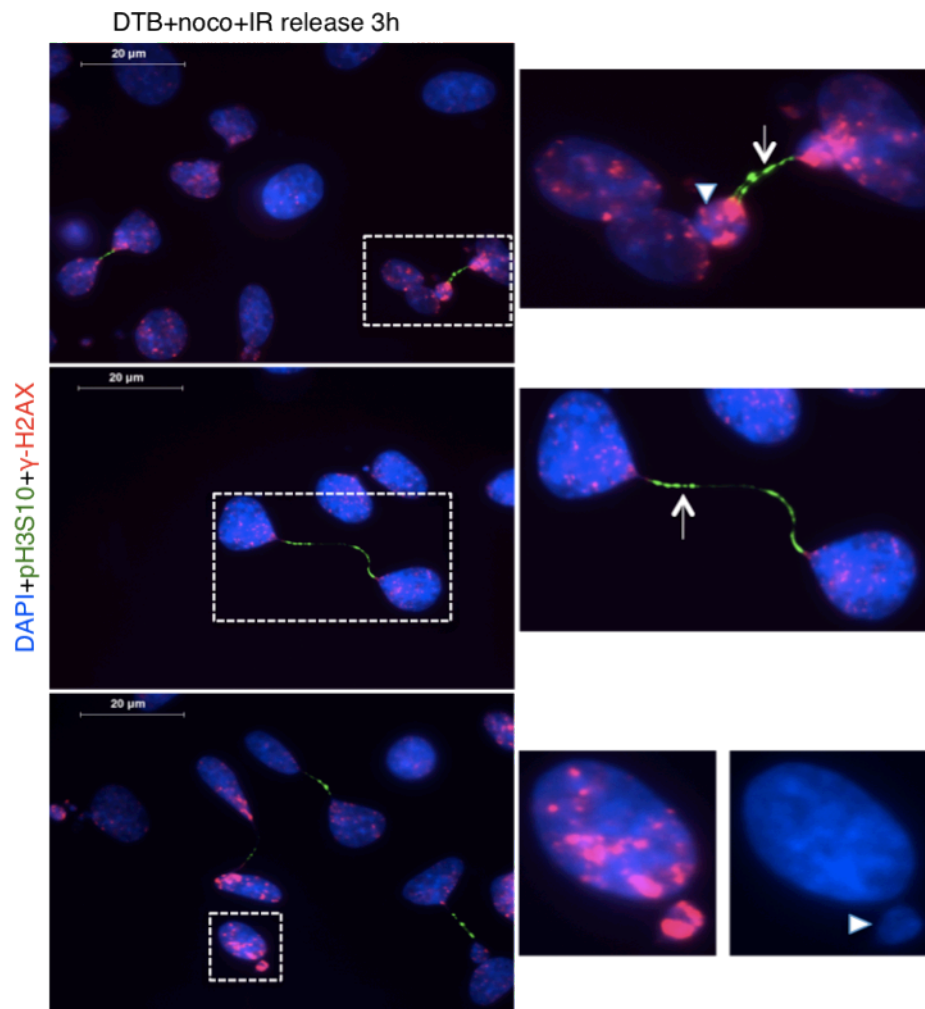


Figure 3- Chromosome segregation defects caused by irradiation of mitotic cells. HeLa cells were synchronized as in Fig. 2D and fixed for immunofluorescence 3h post release. Arrows depict chromosome bridges and arrowhead depicts micronuclei.

4.2.2. DISCUSSION

Mitotic DNA damage is known to have multiple effects on cell cycle progression. Some studies have shown that cells can revert to an interphase like state (Chow et al., 2003) while others have suggested an arrest in mitosis due to SAC activation (Choi and Lee, 2008), (Mikhailov et al., 2002). We reassessed this issue by following cells that received DNA damage while being in mitosis and that were then released from the mitotic block. To this end, we used either a single nocodazole-treatment approach or a combination of synchronizing agents (DTB together with a brief nocodazole treatment). In particular, using the latter approach we obtained mitotic synchronization by nocodazole avoiding unwanted effects caused by prolonged treatment with the drug, such as mitotic slippage (Brito and Rieder, 2006). In the case of long nocodazole treatment (16h), the results of flow cytometry (Fig. 2C, Control) confirmed that cells were healthy. As a measure of the presence of DNA damage in condensed mitotic chromosomes, we relied on γ -H2AX foci formation. Both, the presence and persistence of these foci clearly indicated sensing of DNA damage and activation of the DNA damage response in mitotic cells (Fig. 1 and 3). However, despite the presence of DNA damage, cells were capable of exiting mitosis as judged by the loss of H3-pS10 signal (Fig. 2A, B) and the distribution of cells in different cell cycle phases, as indicated by the flow-cytometry profile (Fig. 2C). These data suggest that although mitotic cells sense DNA damage, they do not arrest or slow down transition through mitosis, possibly due to the reduced chance of repairing DNA in the highly condensed chromosomes. Much in accordance with our results, a recent study clearly showed that damage induced during mitosis is not addressed at this time of the cell cycle; rather, cells first complete mitosis, and then damage is repaired in the following G1. This is most likely due to impaired access of proteins to the break sites in highly condensed chromatin (Giunta et al., 2010). The persistence of γ -H2AX foci in newly divided cells seen here fully supports the above findings.

As the damaged cells exited mitosis, we attempted to identify possible phenotypes caused by the persistence of unrepaired DNA structures. Interestingly, there was a dramatic increase in the percentage of cells with mis-segregated chromosomes, including lagging chromosomes at metaphase and the presence of DNA bridges at

anaphase; the latter remained even after the cells had decondensed their chromosomes and divided (Fig. 3). Also, the later time points analyzed showed an increased occurrence of micronuclei (Fig. 3). Micronuclei are known to be manifestations of the DNA that form the anaphase bridges and of the whole lagging chromosomes. In our experimental conditions, these chromosomal aberrations could either be a direct effect of DNA damage on the chromosomes or could be at least in part contributed by the loss of functional AurA-TPX2 complex (Results Part 1), suggesting a role of this complex in late mitotic events. Further work, such as introduction of enzymatically active AurA in DNA damaged cells, would allow assessing whether this phenotype can be rescued and, in turn, would provide more insights into the function of the AurA-TPX2 complex in chromosome segregation and cytokinesis.

Clearly, other important issues remain to be addressed, such as the destiny of daughter cells carrying gross chromosomal aberrations. It is conceivable that such cells would arrest in the next G1 in the attempt to repair the damage, or that they continue cycling at the expense of increased aneuploidy or, finally, that they undergo apoptosis. Although purely speculative, the first possibility seems to be the most unlikely: indeed, even if repair is attempted, it may not prove to be beneficial to aneuploid cells that contain micronuclei. On the other hand, the possibility that cells irradiated during mitosis may undergo apoptosis during the following cell cycle is an interesting concept for cancer therapy. Understanding the molecular mechanism that underlies this outcome would provide the rationale for clinical trials where combination of taxanes and radio-mimetic agents have been employed (Choy et al., 1998), (Milas et al., 1999), (Cmelak et al., 2007), a treatment that would be particularly suited in the case of resistance to taxol, as observed in AurA (Scharer et al., 2008), (Anand et al., 2003) and TPX2 (Warner et al., 2009) overexpressing tumors.

4.2.3. CONCLUSIONS

This part of the study shows that administering damage to mitotic cells does not cause cell cycle arrest, as previously suggested by others (Mikhailov et al., 2002), (Choi and Lee, 2008), (Chow et al., 2003). We report that cells are fully able to complete mitosis after irradiation-induced damage, albeit with severe chromosomal segregation defects. A similar observation was made when damage was induced in metaphase cells (Results section, Part 1).

Taken together with the results presented in 1st part of this study, where we show that irradiation of mitotic cells results in reduced AurA kinase activity, we speculate that the chromosomal segregation defects observed in this second part of the study, where the outcome of mitotic cells irradiation was examined, is partially contributed to by the reduced functionality of the AurA-TPX2 complex. These results suggest that the possible connection between inhibition of AurA kinase activity and the appearance of chromosomal aberrations upon irradiation of taxol-treated cells should be explored for its practical implications in cancer therapy.

4.2.4. REFERENCES

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4.2.5. MATERIALS AND METHODS

Antibodies and chemicals

The following antibodies were used: PP1 (Santa Cruz Biotech., mouse monoclonal); H3-pS₁₀ (Millipore; rabbit polyclonal), γ -H2AX (Millipore, mouse monoclonal) and MSH2 (Santa Cruz Biotech., rabbit polyclonal sc-494).

Thymidine (SynGen) was dissolved in PBS and filter-sterilized. Nocodazole (Sigma) and etoposide (Alexis Biochem.) were dissolved in DMSO and stored in aliquots at -20°C.

Cell culture and synchronization

HeLa cells were maintained in DMEM (GIBCO) supplemented with 5% fetal calf serum (FCS, GIBCO), penicillin (10,000 U/ml) and streptomycin (10,000 μ g/ml) (GIBCO) at 37°C and 5% CO₂. Cells were synchronized with double-thymidine block (DTB) release method. Briefly, cells were treated with 2 mM thymidine for 16h after 24h of plating. Cells were washed twice with PBS and released for 8h in fresh medium followed by a 2nd thymidine treatment for 16h. Cells were released in fresh medium and nocodazole (200 ng/ml) was added 7h post-released followed by IR (9 Gy) at 10h post-release. Cells were collected at indicated time points.

HeLa cells were also synchronized by nocodazole alone. Briefly, cells were treated with 200 ng/ml nocodazole for 14h or 400 ng/ml nocodazole for 16h, 24h after plating. Cells were released from nocodazole block and immediately treated with IR and collected at the indicated time points. Where indicated cells were treated with 5 μ M etoposide for 1h before release into fresh medium.

Western blotting

Whole-cell extracts (WCE) were prepared using immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM PMSF, 1% Nonidet P-40). Briefly, cells were washed with cold PBS twice and incubated in the lysis buffer on ice for 5 minutes and then scraped. Cleared lysates were obtained by centrifuging the tubes at 13,000 rpm for 15 minutes at 4°C. Samples were boiled in 1X Laemmli-buffer (50 mM Tris-Cl pH-6.8, 100 mM DTT, 2% SDS,

0.1% BPB dye, 10% glycerol) at 95°C for 5 minutes before loading on SDS-PAGE gel.

Time-lapse microscopy

HeLa cells were grown on 3.5-cm glass-bottom dishes (Mat Tek). Cells were imaged on an Olympus IX81 fluorescence microscope equipped with an external temperature control chamber and CO₂ cylinder set to maintain the cells at 37°C with 5% CO₂. Images were captured every 5 min using a CCD camera (Orca AG, Hamamatsu) using the cellR[®] software (Olympus).

Immunofluorescence

Indirect immunofluorescence was performed with cells grown on glass coverslips. At indicated time points, coverslips were fixed in 100% ice-cold methanol and kept at -20°C until processed. For blocking, coverslips were treated with 3% non-fat milk-PBS solution (15-20 min) at RT with gentle shaking. This was followed by overnight treatment with the primary antibodies in a wet chamber at 4°C. The coverslips were washed twice in 3% non-fat milk-PBS (10 min) with shaking and probed with secondary antibodies (1h) at RT in dark. Washings were done with PBS twice for (10 min) with gentle shaking. The coverslips were then washed with Milli-Q water and mounted on Vecta-shield DAPI (Vector Labs) solution and sealed. Alexa Flour488 and 594 conjugated secondary antibodies from Invitrogen were used at 1:1000 dilution. Cells were observed with a Leica DMRB microscope equipped with a 100W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (HCX PL APO 63X) and images were captured with a Leica DFC 360 FX camera. Merged images were obtained using the Image Overlay plugin of the Leica Application Suite[®] software.

Flow Cytometry

DNA analysis by PI staining was performed to determine the cell cycle distribution on samples collected before and after synchronization. Briefly, cells were trypsinized and resuspended in full medium, counted and transferred to a FACS tube. Cells were pelleted at 1500 rpm for 5 min and washed twice with PBS and fixed in 1ml ice-cold 70% ethanol added drop wise with continuous mixing on a vortex mixer. Samples

were stored at 4°C until processed for analysis. For PI staining, 0.5 to 1×10⁶ fixed cells were pelleted at 1500 rpm to remove ethanol and washed twice with PBS. Cells were resuspended in 500 µL PBS and treated with RNase (100 µg/ml, Sigma) for 30 min at RT. PI (20 µg/ml, Molecular Probes) was added and cells were left on ice in dark for 30 min prior to analysis. 10,000 events were acquired using Dako instrument and analyzed using the Summit software.

PART 3- ONGOING PROJECT

Identification and Functional Evaluation of Novel Interacting Proteins of Aurora A

(Project in collaboration with Dr. Dorothea Rutishauser and Dr. Bertran Gerrits, Functional Genomics Center, University of Zurich)

4.3. IDENTIFICATION AND FUNCTIONAL EVALUATION OF NOVEL INTERACTING PROTEINS OF AURORA A

AIM

AurA is an essential protein kinase required for a plethora of events taking place during the finely tuned process of mitosis. Till date many of its interacting proteins have been identified both from G2 and M-phase, thus enriching our understanding of the G2-M transition and mitotic entry. This part of my thesis was aimed at identifying a set of novel proteins that interact with AurA specifically during mitosis. These could either be substrates of AurA or proteins that act upstream to the kinase regulating its function and/or localization. The final goal was the elucidation of novel signaling pathways involving AurA, responsible for yet to be identified functions during mitosis. In addition, we reasoned that the newly identified proteins could be relevant elements of the process of tumorigenesis, as AurA is a potential oncogene.

4.3.1. RESULTS AND DISCUSSION

We used mass-spectrometry (MS) to identify novel interacting partners of human AurA. Because AurA is regulated by direct interaction with p53, we chose MCF-7, a human breast cancer cell line as the model system that has a functional p53 status.

The experiment was performed twice and selected results from both experiments are presented in the ‘**Tables**’ section. Both MS-experiments were carried out using the same extracts, the difference being, for the 1st experiment, extracts were not pre-cleared with beads before IP was performed. Consequently, the list obtained contained many proteins non-specifically binding to the beads. The 2nd experiment was thus an improved version of the 1st one.

4.3.1.1. IDENTIFICATION OF NEW INTERACTION PARTNERS OF AURORA A

The following steps were performed:

4.3.1.1.1. Synchronization of cells

To obtain a mitotic population of cells, where both AurA kinase activity and protein level peak during the cell cycle, we carried out cell synchronization. Cdk1 is an important kinase required for entry into mitosis and blocking its activation results in arrest of cells in G2 phase. We employed an inhibitor, RO-3306, previously described to specifically inhibit Cdk1 and arrest cells at the G2-M boundary (Vassilev et al., 2006). Following the treatment, cells were released into fresh medium without the drug and synchronization was tested at the indicated time points using flow-cytometry (Fig. 1A) and Western blotting approach (Fig. 1B). At 0h post-release we detected a reduced phosphorylation at T₃₂₀ in PP1, a Cdk1 phosphosite (Wu et al., 2009), indicating inhibition of Cdk1. PP1 was phosphorylated again upon release from the inhibitor, indicating re-activation of Cdk1 and mitotic entry. Cells were purely mitotic approximately 15-30 min post-release with maximum level of AurA protein (and other mitotic proteins: TPX2 and cyclin B1) and highest level of PP1 and histone H3 phosphorylations present (Fig. 1B). This synchronization approach, in addition, allowed us to detect microtubule interacting proteins as the spindles were kept intact, unlike those resulting from nocodazole treatment.

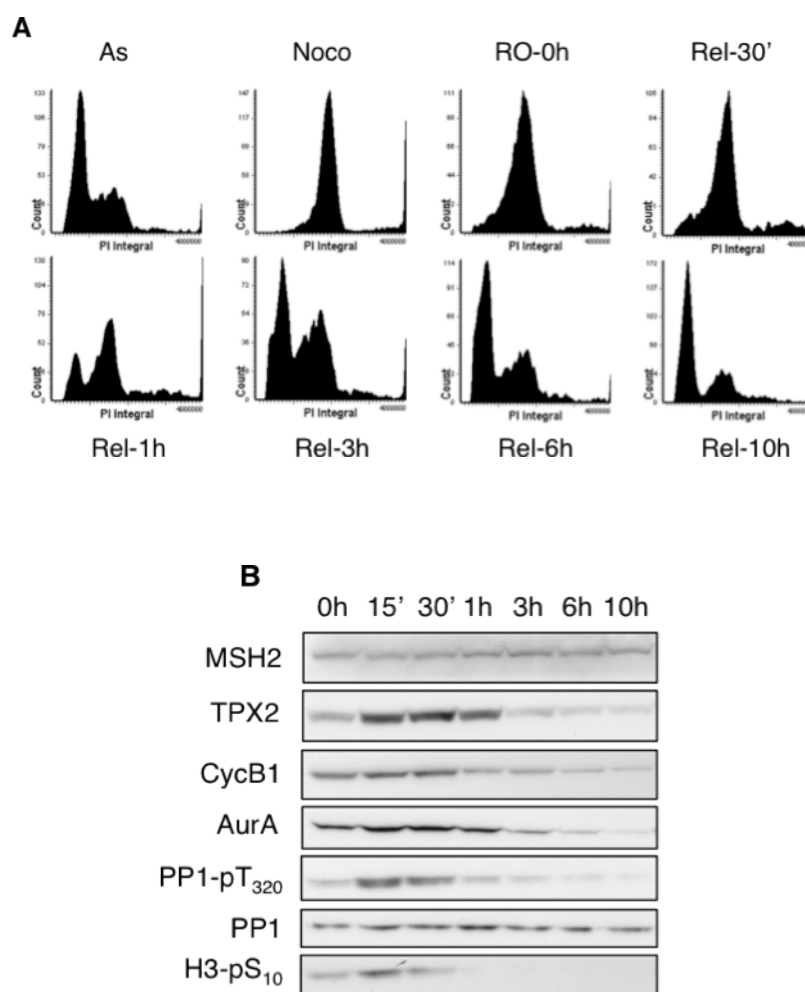


Figure 1- Synchronization of MCF-7 cells with RO-3306. A) MCF-7 cells were treated with 9 μ M RO-3306 for 20h, released in fresh medium without the drug and harvested at indicated time points for flow-cytometry analysis. As: asynchronous, Noco: treatment with 400 ng/ml nocodazole for 16hrs, used as a positive control. B) WCE prepared from cells treated as in a) were run on SDS-PAGE gel and proteins were detected by Western blotting using specific antibodies.

4.3.1.1.2. Immunoprecipitation of AurA and Silver Staining

In order to identify novel interacting proteins, AurA was immunoprecipitated using a specific antibody from WCE of RO-3306 synchronized cells, as mentioned above. An equal amount of pre-immune serum was used for carrying out the control

immunoprecipitation (IP). Immunoprecipitates were run on an 8% SDS-PAGE gel and visualized using MS-compatible silver staining procedure as shown in Fig 2.

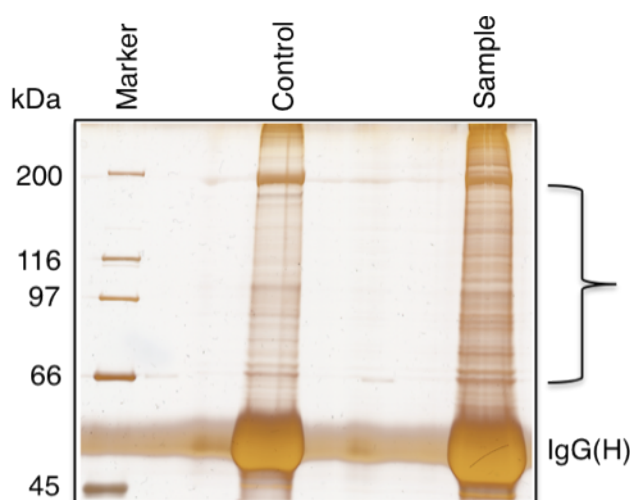


Figure 2- Silver stained gel showing proteins seen after immunoprecipitation of *AurA* with pre-immune serum (Control) and specific antibody (Sample). Bands in the region marked with a bracket (between 200 kDa and 66 kDa) were excised from control and sample lanes and in-gel trypsinization was performed (see Materials and Methods).

4.3.1.1.3. Proteins identified by mass-spectrometry

Bands were carefully excised from the gel as described in Materials and Methods and further processed before injecting into the MS-instrument. The raw data obtained was used for performing a BLAST search against a human protein database (SwissProt) to identify the proteins. **Table 1** shows a selection of proteins identified by the 2nd experiment that were considered most significant depending upon the number of unique peptides. **Table 2** shows the most interesting mitotic proteins shortlisted from Table 1. **Table 3** shows candidate proteins identified from the 1st MS-experiment (presented here using the same criterion like in Table 1). **Table 4** lists proteins that were identified in both the screens.

4.3.1.2. SELECTION AND VALIDATION OF IDENTIFIED PROTEINS

4.3.1.2.1. Primary Screening

Of the list of proteins obtained, a primary selection was made based on three important criteria: first, number of unique peptides (Table 1) and proteins that appeared in both the screens (Table 4); second, relevance of identified proteins to cell cycle and mitosis; and third, novelty of the proteins. The preliminary screening strategy used to validate the selected candidates was to perform their downregulation in asynchronous cells using RNAi and identify any defects in mitotic entry and/or progression. We relied on two technical read-outs for this. **First**, flow-cytometric analysis was used to determine the distribution of cells in different phases of cell cycle after downregulation. **Second**, fluorescence microscopy was employed to visualize any mitotic defects arising due to downregulation. The following parameters were scored: defects in entry into mitosis, spindle defects (monopolar spindles, abnormal bipolar spindle apparatus), problems in chromosome compaction, alignment and segregation (misaligned metaphases, anaphase bridges) and defective cytokinesis (binucleate and multinucleate cells). Any defects observed would indicate a direct role of the protein in G2-M transition and/or in regulation of the mitotic process.

The result of this primary screening is presented below. As a general rule, two siRNA oligonucleotides were tested for each target. Only oligonucleotides validated by the manufacturer (Qiagen) were used in single (AurA, Anillin and SIN3A).

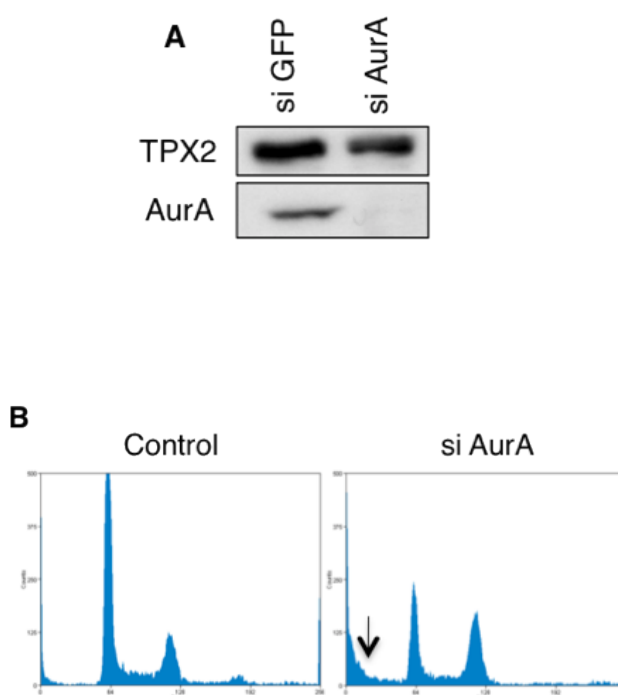
Stable HeLa cell line expressing mGFP-tubulin and mCherry-red-H2B plasmids was used for the primary screening experiments, referred to as HeLa* throughout this section.

4.3.1.2.1.1. Effect of AurA downregulation on cell cycle progression

4.3.1.2.1.1.1. As a control for the preliminary screening, we first analyzed the effect of loss of AurA on cell cycle progression and mitosis. AurA was downregulated in HeLa* cells and protein expression was examined by Western blotting 48h after transfection. The siRNA oligonucleotide used successfully downregulated AurA from

HeLa* cells (Fig. 3A). Next, the effect of AurA downregulation was tested by flow-cytometry. As shown in Fig. 3B, a significant accumulation of cells in G2-M phase and increase in the percent of apoptotic cells (seen as the sub-G1 population indicated with an arrow) was observed when compared to the control.

Upon direct visualization of cells by fluorescence microscopy, a significant increase in the percentage of mitotic cells (scored by counting cells with condensed chromosomes) was found (Fig. 3C, upper panel), indicating cell cycle arrest. In addition, most of the mitotic cells had defective centrosome separation (evident by increased occurrence of cells with monopoles) and showed lack of spindle bipolarity and normal spindle apparatus (Fig. 3C, lower panel). Also, there were defects in chromosome compaction and almost no metaphases were seen, indicating that cells were blocked from progressing further in mitosis. These results confirm published evidence and indicate that AurA is an essential kinase required for centrosome separation, formation of bipolar spindle and normal mitotic progression. We assumed that if a protein that interacts with AurA plays a role during mitosis, its downregulation might also result in similar phenotypes.



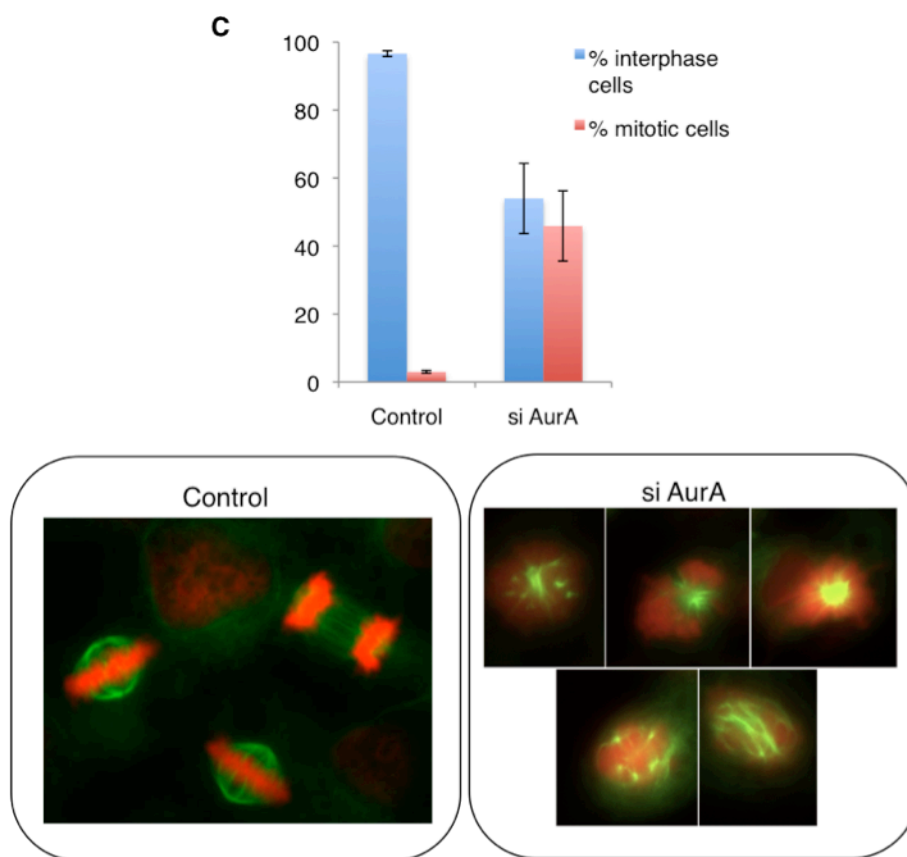


Figure 3- Effects of AurA downregulation. A) HeLa* cells were transfected with 10 nM AurA siRNA oligonucleotide and analyzed by Western blotting 48h later. B) Cells were treated as in a) and fixed for flow-cytometric analysis. Ungated profiles are shown to evidence the presence of a sub-G1 population of cells upon RNAi treatment. C) Bar graph showing percent of mitotic and interphase cells in control and siRNA treated samples; fluorescence images showing mitotic figures from the same.

4.3.1.2.1.1.2. We further tested the effect of AurA downregulation on cell cycle progression in synchronized cells. HeLa* cells were first transfected with AurA siRNA oligonucleotide and 24h later synchronized with a single thymidine block. Cells were analyzed by flow-cytometry at the indicated time points. As anticipated, cells with no siRNA were mitotic at 10h post release and started entering the subsequent G1 at 12h (Fig. 4A). In contrast, cells in which AurA was downregulated remained in G2-M even at 12h post release, indicating that cells were blocked from progressing further into mitosis. Also, the downregulation was toxic to the cells as there was a significant increase in apoptotic cells as compared to the control (Fig.

4B), confirming the data obtained from downregulation in asynchronous cells (Fig. 3B). Together these results confirmed the previously established function of AurA in mitotic progression.

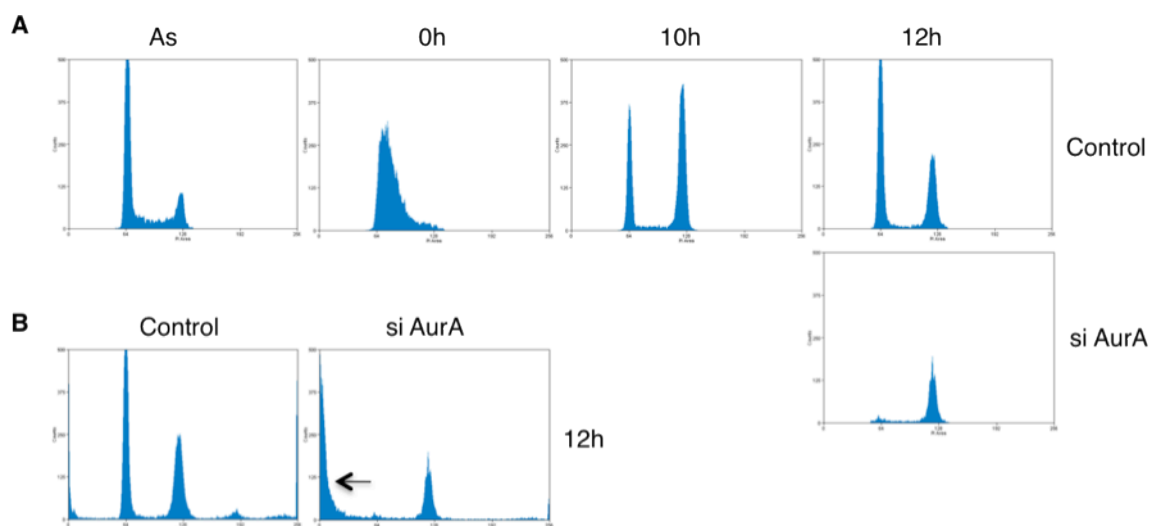


Figure 4- Effect of AurA downregulation on synchronized cells. A) *HeLa** cells were transfected with 10 nM AurA siRNA oligonucleotide and 24h later 2 mM thymidine was added to the cells for another 24h. Cells were released from the thymidine block into fresh medium and collected for flow-cytometry at indicated time points. B) Flow-cytometry profiles of a) were ungated to evidence the sub-G1 population, shown with an arrow.

4.3.1.2.1.2. Effect of downregulation of selected candidates

The following proteins were shortlisted by the above-mentioned criteria and were tested by siRNA-mediated downregulation approach.

4.3.1.2.1.2.1. SMARCA4 (SWI/SNF-related Matrix-associated Actin-dependent Regulator of Chromatin subfamily A member 4)/ BRG1

SMARCA4 is an ATPase associated with the SWI/SNF chromatin-remodeling complex. The protein is composed of several highly conserved domains (Fig. 5) and is an important regulator of transcription.

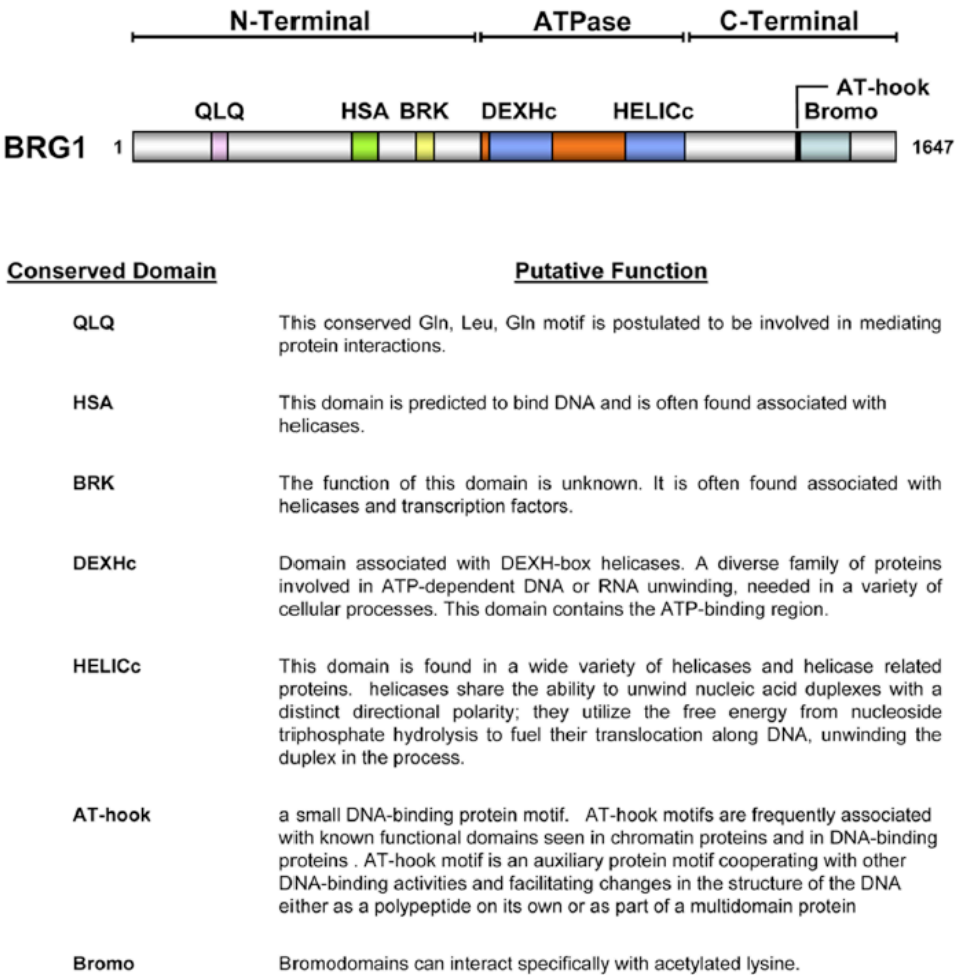


Figure 5- Domain organization of SMARCA4. (Adapted from Trotter and Archer, 2008)

SMARCA4 has been associated with functions like transcriptional activation/repression, DNA repair, replication and recombination as it is a part of a number of protein complexes that regulate the chromatin (Trotter and Archer, 2008). SMARCA4 is also a known regulator of cell proliferation. It interacts with pRb tumor

suppressor protein and regulates transcription of genes required for S-phase entry, e.g., cyclin A and Cdk1 (Wong et al., 2000). Different large-scale proteomics studies showed that the protein is a target of ATM/ATR kinases upon DNA damage (Matsuoka et al., 2007), indicating its potential role is DNA repair processes.

So far its role during mitosis is not known. We investigated its significance by employing RNA interference. SMARCA4 was downregulated from asynchronous HeLa* cells using two different siRNA oligonucleotides and cells were analyzed by flow-cytometry and fluorescence microscopy 48h post-transfection. As shown in Fig. 6A, we did not observe cells accumulating in any one phase of the cell cycle, the distribution being similar to the control. There was, however, a slight increase in 8N population of cells (indicated by arrows), likely reflecting defects in cytokinesis.

As observed by fluorescence microscopy, cells displayed many aberrant mitotic phenotypes (Fig. 6B). Cells had severe defects in chromosome compaction and alignment at the metaphase plate and although bipolarity was achieved, cells did not have a normal spindle apparatus. Occasionally, occurrence of binucleate cells (marked with white arrows) was seen, indicating problems in cytokinesis.

Similar results in flow-cytometry and mitotic defects were obtained with both siRNA oligonucleotides confirming the specificity of downregulation. These preliminary data point towards a role of SMARCA4 in progression through rather than entry into mitosis.

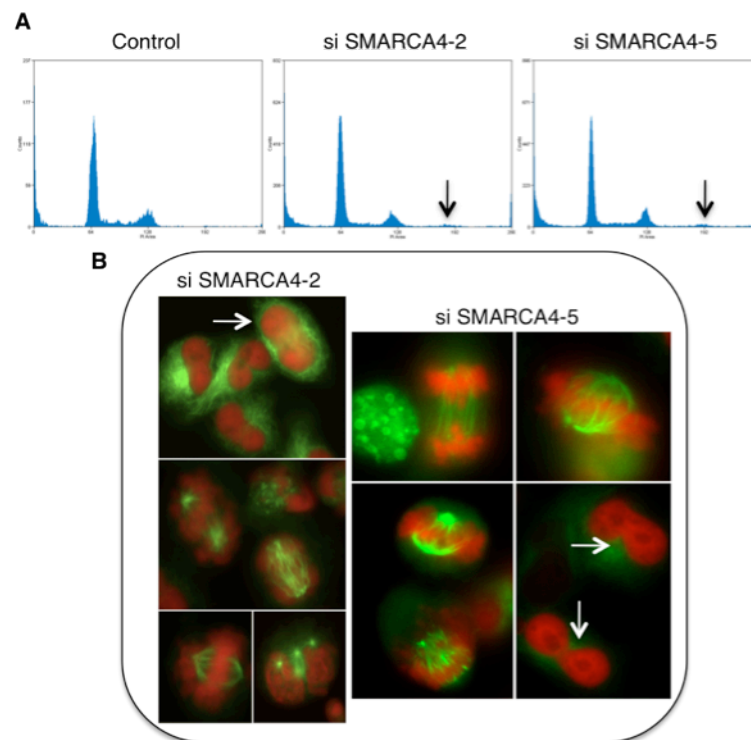


Figure 6- Effects of SMARCA4 downregulation. *HeLa** cells were transfected with 10 nM SMARCA4 siRNA oligonucleotide and analyzed by A) flow-cytometry and B) fluorescence microscopy.

4.3.1.2.1.2.2. ANILLIN

Anillin is an actin-binding protein required for cytokinesis, specifically for the processes of furrow assembly, ingression (Oegema et al., 2000) and maintenance of the contractile ring structure through regulating localization of RhoA and myosin II (Zhao and Fang, 2005). Anillin is also cell cycle regulated like other mitotic proteins and is a substrate of the APC/Cdh1 E3 ligase complex (Zhao and Fang, 2005).

In our experiment, downregulation of anillin did not result in any significant increase in binucleate cells as expected from the data already published (Oegema et al., 2000). There was, however, only a slight increase in 8N and 16N population of cells (Fig. 7A, indicated by arrows). As shown by the fluorescence images (Fig. 7B), there were not many binucleate cells and also cytokinesis appeared to be normal. The explanation could be that the siRNA oligonucleotide used, either, did not work or the

concentration used did not sufficiently downregulate anillin to produce a phenotype. Further work using higher concentration of the siRNA oligonucleotide or different oligonucleotides will be required to test if the downregulation results in any mitotic phenotypes. In addition, RT-PCR will be performed to check the efficacy of the oligonucleotides employed. So far, we did not find any dramatic effect of anillin downregulation.

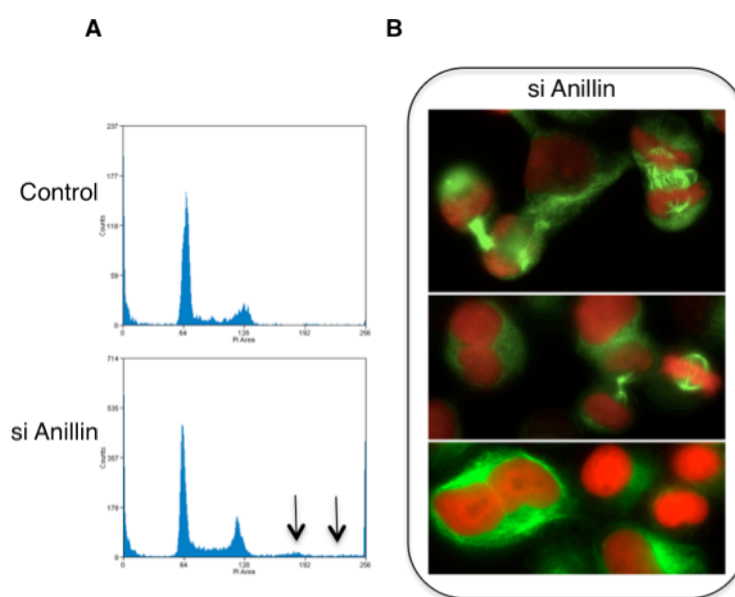


Figure 7- Effects of anillin downregulation. A) HeLa* cells were transfected with 10 nM anillin siRNA oligonucleotide and analyzed by flow-cytometry and B) fluorescence microscopy.

4.3.1.2.1.2.3. SIN3A

SIN3A gene product is a transcriptional co-repressor that was identified initially by its interaction with Myc family of transcription repressors and later found to interact with histone deacetylases (HDACs) forming large protein complexes (Dannenberg et al., 2005). The SIN3A-HDAC complex regulates transcription by associating with a large number of transcription factors, such as p53 (Murphy et al., 1999) and Smad4, resulting in repression of their target genes. Deregulation of transcription by this

complex has been associated with cancers such as AML (Melnick and Licht, 2002). SIN3A is also annotated as a phosphoprotein and mitosis specific phosphorylation sites have been mapped (Dephoure et al., 2008). Its direct role in mitosis, however, is not known.

For evaluation of its role in cell cycle progression, we performed siRNA-mediated downregulation of SIN3A. As shown in Fig. 8A, downregulation of SIN3A from asynchronous cells resulted in an apparent cell cycle arrest as the population of cells in S and G2 phase appeared to be significantly reduced as compared to the control. Whether or not there was an extended block in cell proliferation was not addressed at this time. Also, there was an increase in sub-G1 population (pointed by the arrow), indicating its requirement for cell survival. By direct observation of the cells using fluorescence microscopy (Fig. 8B), we observed a clear reduction of mitotic cells (Fig. 8B, lower panel), supporting the flow-cytometry profile. Although, a few mitotic cells were seen, they did not display any aberrant phenotypes (Fig. 8B, upper panel). These results indicate that SIN3A may be required for entry into mitosis rather than having a role in mitotic progression. Future experiments will aim at uncovering the significance of its interaction with AurA. It will be interesting to determine if SIN3A plays a role in the transcriptional regulation of AurA, if it regulates protein stability or if it has a more direct role in regulating AurA specific mitotic functions, especially entry into mitosis.

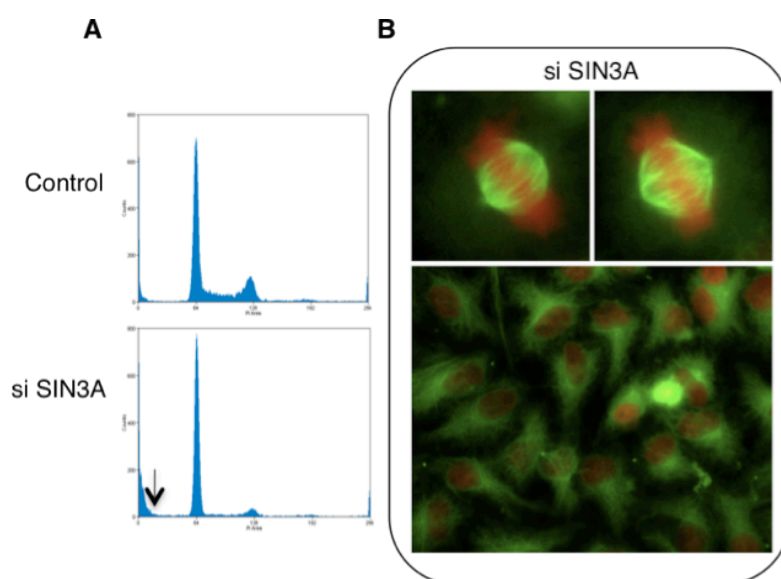


Figure 8- Effects of SIN3A downregulation. *HeLa** cells were transfected with 10 nM SIN3A siRNA oligonucleotide and analyzed by A) flow-cytometry and B) fluorescence microscopy.

4.3.1.2.1.2.4. Cdc5L (Cell Division Cycle 5 Like protein)

The Cdc5L protein is the human homolog of *S.pombe* Cdc5 protein involved in mitotic entry. Cdc5L is associated with pre-mRNA splicing and is also able to bind DNA in a sequence specific manner, indicating a role in transcription regulation (Lei et al., 2000). Overexpression of Cdc5L was shown to shorten G2 and reduce cell size, whereas expression of a dominant negative mutant resulted in delayed entry into mitosis (Bernstein and Coughlin, 1998). The protein thus provides a remarkable example of how transcription regulation and mitosis can be linked; the exact mechanism, however, remains elusive. As we found Cdc5L interacting with AurA, we asked whether Cdc5L might exert its mitotic specific functions by regulating AurA. As an alternative, Cdc5L could be an AurA substrate, since it was reported to undergo heavy phosphorylations during mitosis (Dephoure et al., 2008).

Downregulation of Cdc5L was performed to understand its role in cell cycle progression. As seen in Fig. 9A, siRNA-mediated downregulation of Cdc5L resulted in a decrease in the G2-M peak as compared to the control. Also, there was an increase in sub-G1 population indicating that it is an essential protein. Direct visualization of cells by fluorescence microscopy (Fig. 9B) revealed that there were fewer mitotic cells (Fig. 9B, lower panel labeled with *) and those that managed to enter mitosis (may be due to incomplete downregulation) showed aberrant mitotic phenotypes. Clear defects in chromosome compaction, chromosome alignment and bipolar spindle assembly were seen with both the siRNA oligonucleotides tested, indicating that the effects are specifically resulting from Cdc5L downregulation. In addition, the data suggest that Cdc5L is directly involved in mitotic entry and progression. It will, therefore, be very interesting to identify its role during mitosis with respect to AurA. The next immediate goal will be to observe localization of AurA in Cdc5L downregulated synchronized cells.

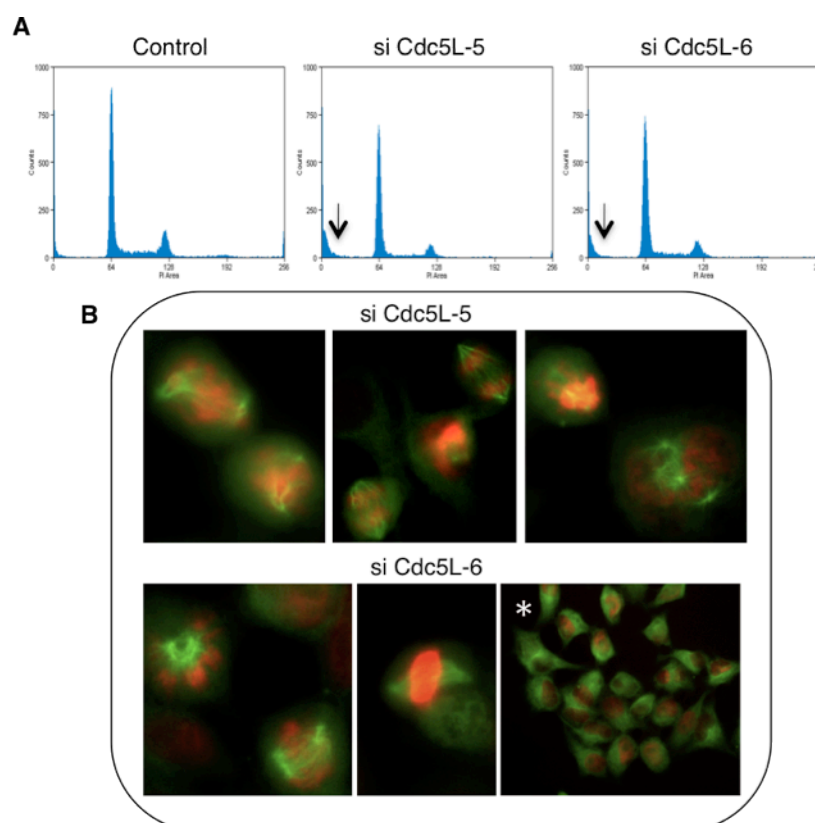


Figure 9- Effects of Cdc5L downregulation. *HeLa** cells were transfected with 10 nM Cdc5L siRNA oligonucleotide and analyzed by A) flow-cytometry and B) fluorescence microscopy.

4.3.1.2.2. Secondary Screening

Here, we tested some significant proteins identified by the 2nd MS-experiment for their interaction with AurA by immunoprecipitation (IP) and Western blotting approach. Localization of these proteins was tested by immunofluorescence (IF) technique. Downregulation of these (to determine any mitotic phenotypes) were not performed, as data in literature is already available.

4.3.1.2.2.1. SAS-6 (Spindle Assembly Abnormal protein 6 homolog)

SAS-6 is a coiled-coil protein first identified in *C.elegans* required for centriole formation (Dammermann et al., 2004), (Leidel et al., 2005). Its human homolog

HsSAS-6 is also associated with centrosome duplication. Downregulation of HsSAS-6 results in monopolar spindle formation and overexpression leads to a multipolar spindle pattern (Leidel et al., 2005) due to its role in the formation of procentrioles (Strnad et al., 2007). It was also observed that SAS-6 levels are cell cycle regulated, being high in mitosis and degraded in an APC/Cdh1 dependent manner at the end of mitosis (Strnad et al., 2007). Human SAS-6 was identified as a phosphoprotein by a large-scale proteomics study of mitotic phosphorylations (Dephoure et al., 2008). So far, only one kinase has been identified for SAS-6, the Zyg1 kinase, a homolog of human Plk4 that phosphorylates *C.elegans* SAS-6 at S123 (Kitagawa et al., 2009). As both loss and overexpression of SAS-6 results in phenotypes similar to those observed upon deregulation of AurA levels, we decided to investigate the significance of this interaction. The fact that both AurA and SAS-6 are localized at the centrosomes makes their interaction more likely.

In order to evaluate this further, we first reproduced the IP done for mass-spectrometry to confirm AurA-SAS-6 interaction. As seen in Fig. 10, IP of AurA from asynchronous and mitotic WCE resulted in clear co-IP of SAS-6.

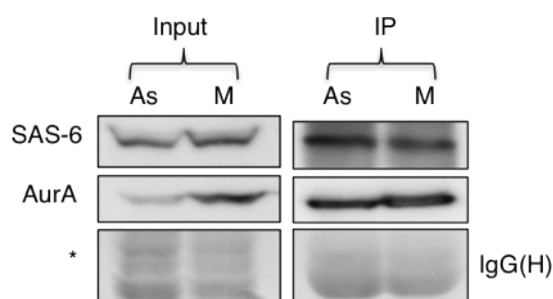


Figure 10- SAS-6 interacts with AurA. AurA was immunoprecipitated from asynchronous (As) and mitotic (M) MCF-7 WCE using specific antibody and interaction with SAS-6 was determined using Western blotting. * and IgG(H) indicate equal loading of input and IP, respectively.

We next examined the cell cycle regulated expression of SAS-6 protein in both MCF-7 and HeLa cells. Cells were synchronized with DTB block and analyzed at the indicated time points. As shown in Fig. 11A, MCF-7 cells were mitotic mainly 8-10h

post release as indicated by phosphorylation of histone H3 and pattern of other cell cycle proteins. SAS-6 also appeared to be specifically high during mitosis and rapidly disappeared as cells entered the next cell cycle (Fig. 11B). A similar result was seen with synchronized HeLa cells (Fig. 11C). We next confirmed the interaction of AurA-SAS-6 from HeLa cells. Surprisingly, although SAS-6 showed a clear cell cycle regulated expression in HeLa cells (Fig. 11C), the two proteins did not interact (data not shown). This could be a result of different p53 status of the two cell lines. Aim of the future studies will be to identify if SAS-6 is a substrate of AurA and test its localization by IF in MCF-7 cells.

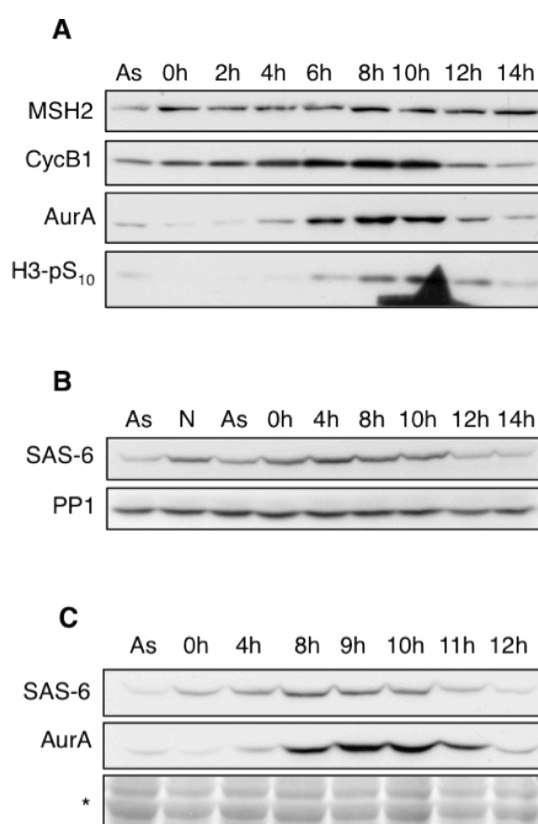


Figure 11- SAS-6 is cell cycle regulated. MCF-7 cells were synchronized by DTB and A) expression of selected mitotic proteins was analyzed at the indicated time points by Western blotting. As: asynchronous. MSH2 serves as the loading control. B) Extracts of a) were used to determine the expression of SAS-6, N: Nocodazole treated sample. PP1 serves as the loading control. C) Similar results were obtained with DTB synchronized HeLa cells. * represents Ponceau-red stained bands showing equal loading.

4.3.1.2.2.2. 53BP1

53BP1, a protein involved in the DNA damage response, was initially identified as an interactor of p53 (Iwabuchi et al., 1994). Since its discovery, it has been shown to interact with a number of proteins directly involved in DNA damage response signaling. 53BP1 co-localizes with damage-induced foci of MRN complex and γ -H2AX (Schultz et al., 2000), (Rappold et al., 2001), (Anderson et al., 2001). Moreover, it is known to be phosphorylated by ATM in response to IR (Rappold et al., 2001), (Anderson et al., 2001), (Xia et al., 2001). Most interestingly, it was found to be hyperphosphorylated during mitosis (Jullien et al., 2002), (Dephoure et al., 2008) with a further increase upon activation of the spindle checkpoint (Jullien et al., 2002), although 53BP1 downregulation did not impair mitotic progression (van Vugt et al., 2010). 53BP1 also associates with the kinetochores, most strongly to prophase and prometaphase chromosomes (Jullien et al., 2002). Thus, so far, 53BP1 has been found associated with mitosis without an identified function.

With evaluation of 53BP1 in this study, we wanted to identify its role in AurA mediated mitotic events and/or in regulation of AurA itself. We first wanted to confirm the interaction of AurA with 53BP1 by IP. We found that the two proteins strongly interacted with each other in mitosis but also a significant interaction was seen in interphase (Fig. 12A). This was possibly due to titration of the antibody by the large amount of WCE used in IP experiments, rendering the antibody unable to detect varying amounts of AurA. This experiment will be repeated using increasing amounts of the antibody and fixed amounts of WCEs. In order to determine if 53BP1 is cell cycle regulated, we synchronized HeLa cells with DTB. The success of synchronization was tested using flow-cytometry (Fig.12B) and WCE obtained from synchronized cells were subjected to Western blot analysis. As shown in Fig. 12C, 53BP1 protein level clearly fluctuated during the cell cycle, being low in S and G2 phases and increased during mitosis and exit from mitosis. The interaction was also confirmed in HeLa cells and it clearly showed a cell cycle dependent pattern (Fig. 12D).

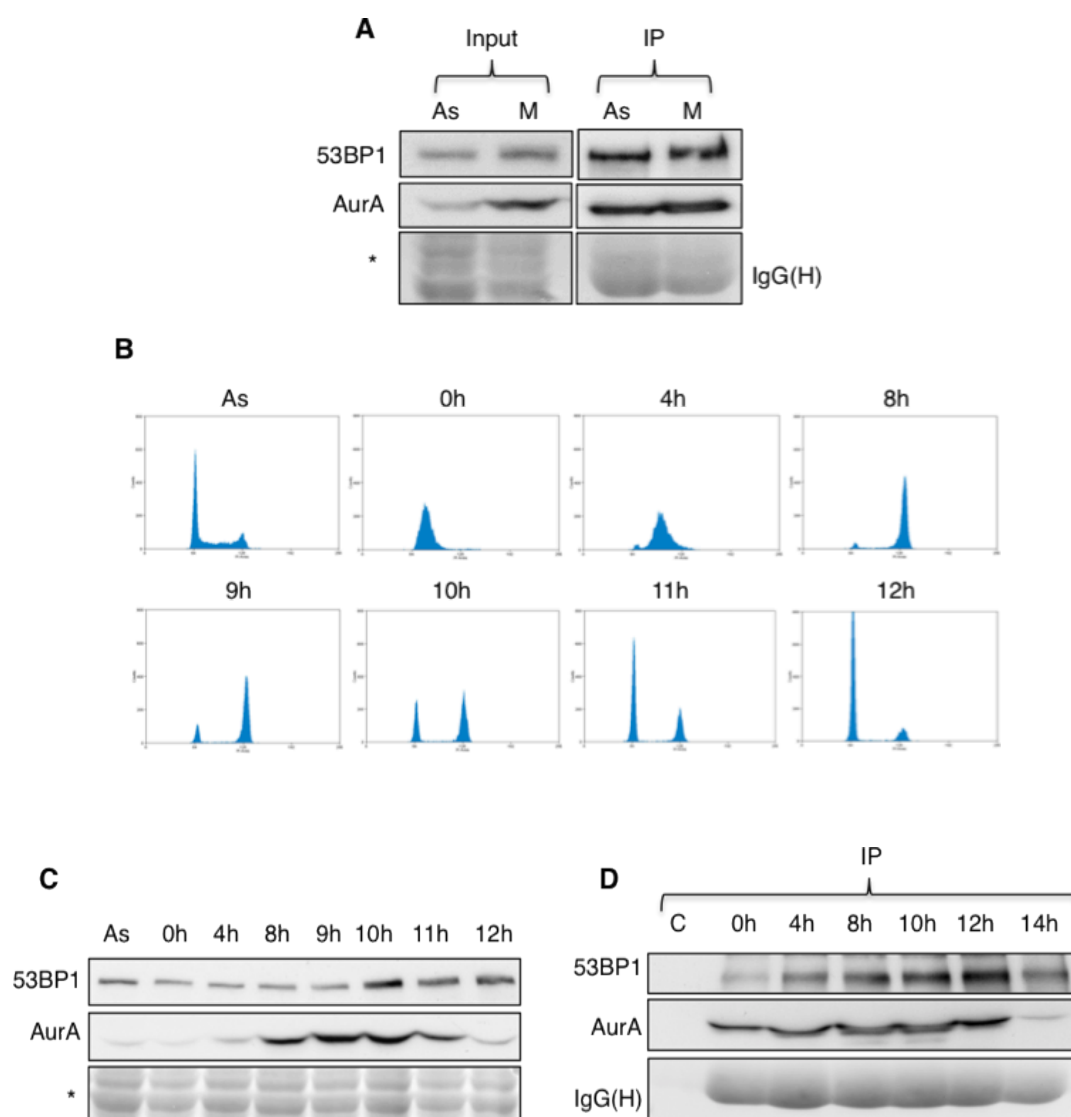


Figure 12- 53BP1 is cell cycle regulated and interacts with AurA. A) AurA was immunoprecipitated from asynchronous (As) and mitotic (M) MCF-7 WCE using specific antibody and interaction with 53BP1 was determined using Western blotting. * and IgG(H), stained with Ponceau-red indicate equal loading of input and IP, respectively. B) Flow-cytometry analysis of HeLa cells synchronized with DTB-release method. DNA was stained with PI. C) DTB synchronized HeLa WCE were subjected to Western blotting and 53BP1 and AurA were detected using specific antibodies. D) AurA was immunoprecipitated from HeLa WCE prepared as in c) and detected using specific antibodies. IgG(H) stained with Ponceau Red serves as loading control for the IP.

Further, we tested if 53BP1 co-localizes with AurA during mitosis. For this, we first tested if the antibody used could detect 53BP1 in both damaged and undamaged conditions. As shown in Fig. 13A, in the absence of damage, 53BP1 primarily showed a diffused staining in the nucleus. Upon IR-induced damage, 53BP1 specific foci were distinctly visible in interphase cells (Fig. 13B). This confirmed the specificity of the antibody. However, there were no foci in the condensed mitotic chromosomes (Fig. 14A), irrespective of the damage (Fig. 14B), as suggested previously (Jullien et al., 2002), (Giunta et al., 2010). Moreover, we could not detect 53BP1 staining in chromosomes that were misaligned during mitosis (Fig. 14A, B), contrary to the already published data that suggested 53BP1 to be kinetochore localized during mitosis and also in misaligned chromosomes (Jullien et al., 2002).

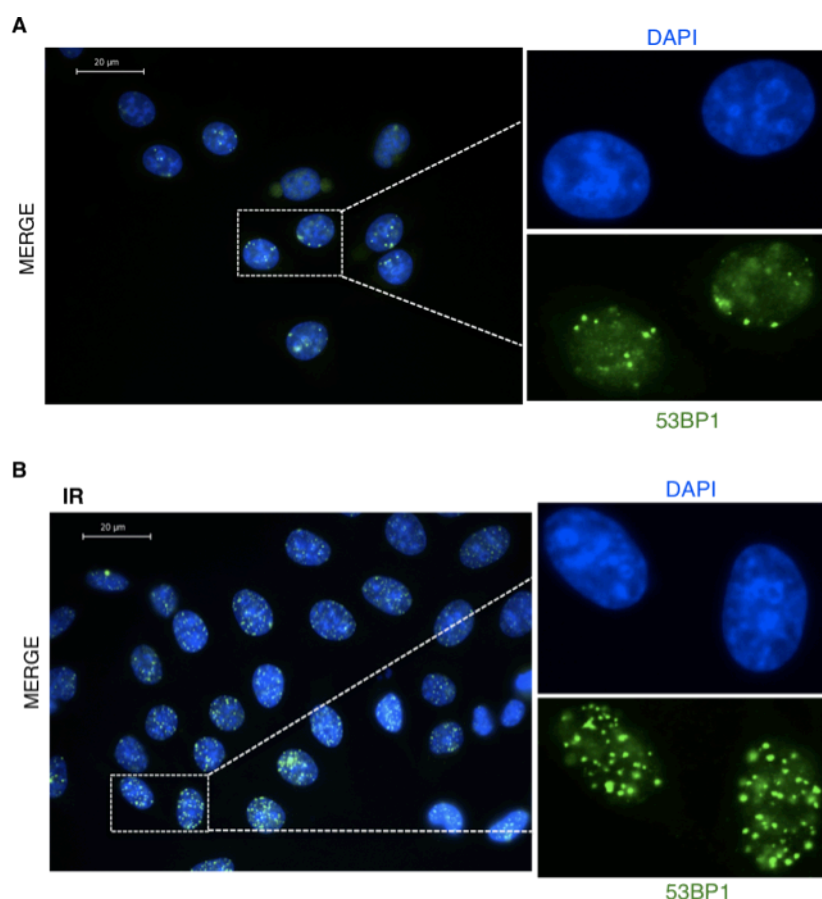


Figure 13-53BP1 forms foci after IR-induced DNA damage. A) Fluorescent image showing 53BP1 staining in non-damaged cells. B) Fluorescent image showing 53BP1 specific foci after IR (9 Gy) induced DNA damage.

Next, we tested its co-localization with AurA, although, by the results obtained above, it was clear that 53BP1 did not associate with mitotic structures such as the centrosomes and spindles, the sites of AurA localization. As anticipated, 53BP1 was not found co-localizing with AurA during mitosis, both in the absence or presence of damage (Fig. 14). A possible explanation could be that their interaction might be happening before cells enter mitosis (Fig. 12D) and continues in mitosis but with distinct AurA population that is not targeted to the centrosomes and mitotic spindles. Identifying AurA and 53BP1 after IP of TPX2 (to determine if 53BP1 interacts with spindle localized AurA or with a distinct AurA pool) would be an indication of this possibility. Work in the future will aim to identify if 53BP1 is a substrate of AurA and if downregulation of 53BP1 has an effect on localization and/or activity of the kinase.

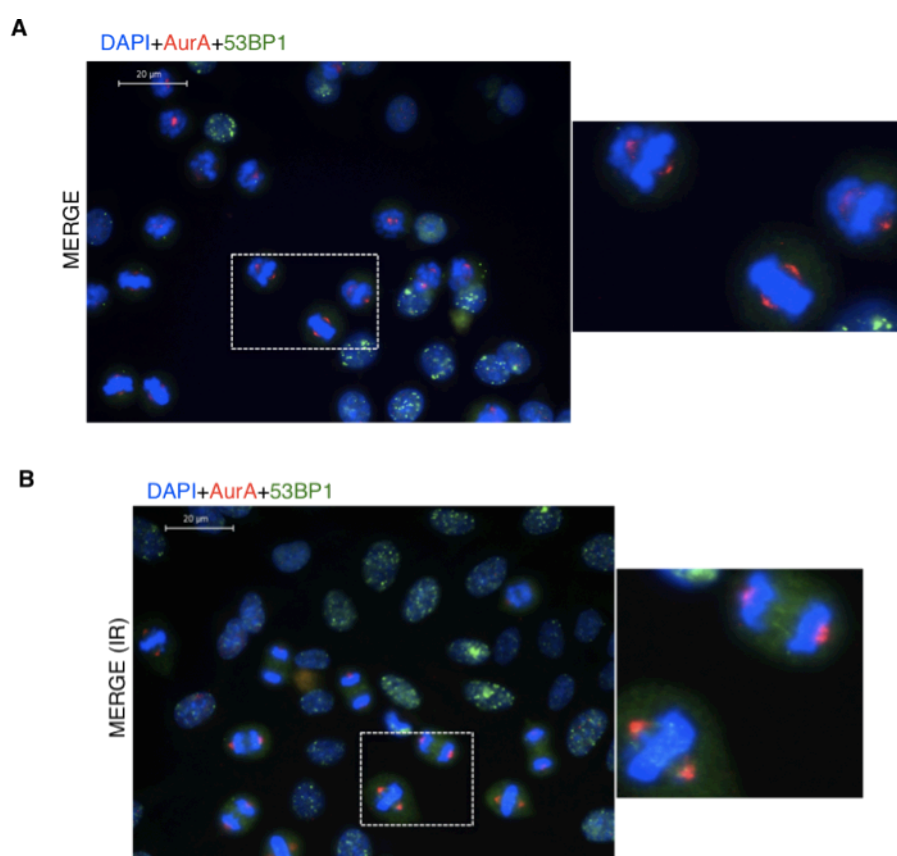


Figure 14- 53BP1 localization during mitosis. A) Fluorescent image showing localization of AurA and 53BP1 during an unperturbed mitosis. B) Fluorescent image showing localization of AurA and 53BP1 after 30 min of IR-treatment (9 Gy) given to DTB synchronized cells at 10h post-release. DNA is stained with DAPI.

4.3.1.2.2.3. KAP-1

KAP-1, also known as KRAB-associated protein 1, is a transcriptional regulator that functions to repress transcription. KAP-1 does not directly bind DNA, but instead acts as a scaffold that binds to proteins containing the KRAB-domain and to chromatin modifying proteins, like HDAC, and represses transcription by allowing chromatin modification to take place (Alter and Hen, 2008). Transcriptional repression by KAP-1 is also mediated through its direct interaction and recruitment of heterochromatin protein 1 (HP1) to the target genes (Ryan et al., 1999). This is suggested to result in local gene silencing by formation of a heterochromatin-like complex within the euchromatin regions, resulting in repression of transcription (Ryan et al., 1999).

KAP-1 is also a significant example of a link between chromatin regulated transcription and DNA damage response as it is phosphorylated by PI3K-like kinases in response to damage and also localizes to the sites of damage (White et al., 2006). During mitosis, chromosomes are maximally condensed thereby making any transcription unlikely. How transcription is regulated at this point is largely unknown.

As we found KAP-1 interacting with AurA, we went on further to characterize the significance of this interaction. KAP-1 was found phosphorylated during mitosis (Olsen et al., 2006), (Beausoleil et al., 2006), (Molina et al., 2007), (Dephoure et al., 2008) and AurA could be one of the kinases responsible for this. We first examined the interaction of the two proteins by IP. As shown in Fig. 15, KAP-1 did not appear to interact with AurA. It also did not seem to be cell cycle regulated as seen by the unvaried level in asynchronous and mitotic WCE inputs. A possible explanation for this finding could reside in the different scale of the two experiments: the large amount of cell extract used for MS might have contained a small amount of a bridging protein the concentration of which is too low in conventional IP experiments to allow detecting the AurA-KAP1 complex. More work will be needed to confirm and clearly establish the significance of this interaction.

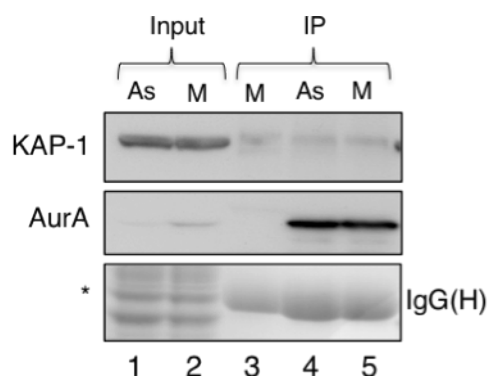


Figure 15- KAP-1 does not interact with AurA. AurA was immunoprecipitated from asynchronous (As) and mitotic (M) MCF-7 WCE using specific antibody and interaction with KAP-1 was determined using Western blotting. * and IgG(H) indicate equal loading of input and IP, respectively. Lane 3 represents control IP from mitotic WCE using pre-immune serum.

4.3.2. CONCLUSIONS

4.3.2.1. Primary screening

AurA downregulation results in increase in the percentage of cells with monoploar spindles and a higher mitotic index, indicating a role in mitotic progression.

Of the proteins analyzed so far, we found that downregulation of SMARCA4 and Cdc5L results in numerous mitotic phenotypes.

SIN3A appears to have some role in entry into the cell cycle rather than mitotic progression.

4.3.2.2. Secondary screening

Proteins for which antibodies were available, such as SAS-6 and 53BP1 were confirmed to directly interact with AurA. 53BP1 was, however, not found co-localizing with AurA during mitosis.

Others, like KAP-1, could not be confirmed as AurA interactor.

4.3.3. OUTLOOK

The goal of future studies will be to first complete the preliminary screening of the selected proteins in Table 1, using a similar approach as used for the others analyzed so far.

Of the proteins analyzed, the aim will be to carry out confirmation experiments for SMARCA4 and Cdc5L, two proteins that appear to have a role in mitotic progression. First, the effect of their downregulation will be tested in synchronized cells to confirm their roles in mitosis. Both flow-cytometry and immunofluorescence (IF) will be employed to this end. The next goal will be to observe the effect of their downregulation on the localization of AurA. Downregulation will be performed in synchronized cells and localization of AurA will be tested by IF. A double knockdown of AurA with either SMARCA4 or Cdc5L will be performed to observe a rescue of the mitotic phenotypes observed. This will clarify if SMARCA4 or Cdc5L directly affect AurA and if they work together with AurA in the same pathway in regulating mitosis.

Their interaction with AurA will be confirmed with techniques such as immunoprecipitation and *in vitro* pull-down experiments, as soon as specific antibodies will be available. Furthermore, the suitability of SMARCA4 and Cdc5L as AurA substrates will be examined.

These preliminary experiments will be necessary to place SMARCA4 and Cdc5L proteins either upstream or downstream to AurA and help in understanding their role as components of AurA pathways.

Since SAS-6 is a known regulator of centrosomes and 53BP1 localizes at kinetochores, understanding their roles in AurA regulated processes will be a novelty. It will be necessary to analyze if these proteins serve as AurA substrates. An interesting issue to examine will be whether AurA mediated phosphorylation plays any role in removal of 53BP1 from the condensed chromatin during mitosis. A similar approach consisting of protein downregulation and cell synchronization will be employed to test if loss of either protein results in AurA mislocalization or vice-versa.

Another interesting family of proteins identified by MS is that of the motor proteins. We found several KIF (Kinesin-like proteins) proteins that associate with the spindle microtubules during mitosis and contribute to the dynamic behavior of spindles. KIF11 or Eg5 is a known AurA interactor and substrate. Identifying more such proteins will be absolutely essential to further understand the role of AurA in regulating motor proteins during spindle formation and maintenance.

We also found two novel centrosomal proteins, Cep192 and Cep170. Cep192 was found associating with AurA also by another MS-study (<http://mitocheck.org>). It will therefore be intriguing to analyze these proteins and find new pathways regulating AurA or being regulated by AurA during mitosis.

A similar methodology will be employed for testing all other proteins that will show mitotic phenotypes (after completion of the primary screening) and interaction with AurA.

4.3.4. REFERENCES

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4.3.5. MATERIALS AND METHODS

Antibodies and chemicals

The following antibodies were used: PP1 (Santa Cruz Biotech, mouse monoclonal); H3-pS₁₀ (Millipore, rabbit polyclonal), AurA (Pab 35, purified rabbit polyclonal; 35C1, mouse monoclonal), 53BP1 (Santa Cruz Biotech, rabbit polyclonal), KAP-1 (Santa Cruz Biotech, rabbit polyclonal), MSH2 (Santa Cruz Biotech, rabbit polyclonal), cyclin B1 (Upstate Biotech. Inc., mouse monoclonal). SAS-6 polyclonal antibody was a kind gift from Prof. Pierre Gonczy ISREC, Lausanne.

Thymidine (SynGen) was dissolved in PBS and filter-sterilized. Trypsin (Promega) was dissolved in 25 mM ammonium bicarbonate and stored as aliquots at -20°C.

Cell culture, synchronization and transfection

HeLa* cell line stably expressing mGFP-tubulin and mCherry-H2B plasmids was kindly provided by Prof. Daniel Gerlich, Institute of Biochemistry, ETH, Zurich. The cells were maintained in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO), penicillin (10,000 U/ml), streptomycin (10,000 µg/ml) (GIBCO) and kept under selection with G418 and puromycin at 37°C and 5% CO₂. Normal HeLa cells were maintained in DMEM (GIBCO) supplemented with 5% fetal calf serum (FCS, GIBCO) and penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) (GIBCO) at 37°C and 5% CO₂. MCF-7 cells were maintained in MEM (GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO), 1% L-glutamine (GIBCO), penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) (GIBCO) at 37°C and 5% CO₂.

MCF-7 cells were synchronized in mitosis using Cdk1 inhibitor, RO-3306, treatment and release method. Briefly, cells were treated with 9 µM RO-3306 for 20h and released into fresh medium. MCF-7 cells were synchronized in G1/S phase by a double-thymidine block (DTB). Cells were treated with 2 mM thymidine (SynGen) for 16h after 24h of plating. Cells were washed twice with PBS and released for 9h in fresh medium followed by a 2nd thymidine treatment for 16h.

HeLa* cells were synchronized with a single thymidine block. Briefly, cells were grown in 10-cm dishes and treated with 2 mM thymidine for 24h. Cells were released in fresh medium and harvested at different time points for western blot and flow

cytometry analysis. Normal HeLa cells were synchronized with DTB as mentioned above.

For transfection, HeLa* cells were seeded in 6-cm dishes and immediately transfected with 10 nM siRNA oligonucleotides (Qiagen) using the Lipofectamin RNAiMAX reagent (Invitrogen) for 48h.

Western blotting and Immunoprecipitation

WCE were prepared using immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM PMSF, 1% Nonidet P-40). Briefly, cells were washed with cold PBS twice and incubated in the IP buffer on ice for 5 minutes and then scraped. Cleared lysates were obtained by centrifuging the tubes at 13,000 rpm for 15 minutes at 4°C. For immunoprecipitations, the WCE were pre-cleared using Protein A Sepharose beads (50% slurry made in 50 mM Tris-HCl, pH 7.5) for 2h at 4°C. AurA was immunoprecipitated with purified AurA-Pab35 antibody for 3h at 4°C from the pre-cleared WCE. IP was carried out in the presence of 50 µg/ml EtBr. Proteins were immobilized on Protein A Sepharose beads (50% slurry made in 50 mM Tris-HCl, pH 7.5) for 2h at 4°C and washed 3 times with 1ml ice-cold IP Buffer. The samples were boiled in 1X Laemmli-buffer (50 mM Tris-Cl pH-6.8, 100 mM DTT, 2% SDS, 0.1% BPB dye 10% glycerol) at 95°C for 5 minutes before loading on SDS-PAGE gel.

Flow Cytometry

DNA analysis by PI staining was done to determine different cell cycle stages on samples collected before and after synchronization. Briefly, cells were trypsinized and resuspended in full medium, counted and transferred to a FACS tube. Cells were pelleted at 1500 rpm for 5 min and washed twice with PBS and fixed in 1ml ice-cold 70% ethanol added drop wise with continuous mixing on a vortex mixer. Samples were stored at 4°C until processed for analysis. For PI staining, 0.5 to 1×10⁶ fixed cells were pelleted at 1500 rpm to remove ethanol and washed twice with PBS. Cells were resuspended in 500 µL PBS and treated with 100 µg/ml RNase (Sigma) for 30 min at RT. 20 µg/ml PI (Molecular Probes) was added and cells were left on ice in

dark for 30 min prior to analysis. 10,000 events were acquired using Dako instrument and analyzed using the Summit software.

Immunofluorescence

Indirect immunofluorescence was performed with cells grown on glass coverslips. At indicated time points, coverslips were fixed in 100% ice-cold methanol and kept at -20°C until processed. For blocking, coverslips were treated with 3% non-fat milk-PBS solution (15-20 min) at RT with gentle shaking. This was followed by overnight treatment with the primary antibodies in a wet chamber at 4°C. The coverslips were washed twice in 3% non-fat milk-PBS (10 min) with shaking and probed with secondary antibodies (1h) at RT in dark. Washings were done with PBS twice for (10 min) with gentle shaking. The coverslips were then washed with Milli-Q water and mounted on Vecta-shield DAPI (Vector Labs) solution and sealed. Alexa Flour488 and 594 conjugated secondary antibodies from Invitrogen were used at 1:1000 dilution. Cells were observed with a Leica DMRB microscope equipped with a 100W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (HCX PL APO 63X) and images were captured with a Leica DFC 360 FX camera. Merged images were obtained using the Image Overlay plugin of the Leica Application Suite[®] software.

Silver staining

MS compatible silver staining procedure was used. Briefly, the gel was fixed in solution A (50% methanol, 5% acetic acid in water, 20 min), washed in solution B (50% methanol in water, 10 min) and rinsed in water two times (10 min) to remove the remaining acid. The gel was sensitized in solution C (0.02% sodium thiosulfate, 1 min) and rinsed with water two times (1 min). This was followed by incubation in solution D (cold 0.1% silver nitrate solution, 20 min) in dark. The gel was developed in a fresh tray using solution E (0.04% formalin in 2% sodium carbonate) with intensive shaking. Once the desired intensity was reached, the gel was transferred in a fresh tray and developing was stopped with solution F (5% acetic acid). Solution F was changed a couple of times to ensure complete termination of developing. All steps were carried out at room temperature. The gel was stored at 4°C in solution G (1% acetic acid) until processed further.

In-gel trypsin digestion of proteins

After silver staining, bands were excised from the gel using a clean blade into eppendorf tubes and destained with solution G (1% H_2O_2). The pieces were dehydrated three times using solution H (50% acetonitrile) followed by reduction of proteins using solution I (10 mM DTT in 25 mM ammonium bicarbonate, 45 min) at 56°C. Alkylation of cysteines was carried out at room temperature by incubating the gel pieces in solution J (50 mM iodoacetamide in 25 mM ammonium bicarbonate, 1h) followed by washing with solution H two times. The gel pieces were dried in speed-vacuum and incubated with solution K (25 mM ammonium bicarbonate) containing trypsin (200 ng, 10 min) followed by addition of a small volume of solution K. The tubes were incubated overnight at 37°C. Tubes were centrifuged to collect liquid from the top and solution L (50% acetonitrile in 5% tri-flouro acetate) was added to the tubes. The supernatant was collected in fresh tubes and this step was repeated. The supernatant was dried using speed-vacuum and tubes were stored at -80°C until processed for mass-spectrometry.

5. TABLES

(From Results Part 3)

5.1. MS-TABLES

Most interesting MS-identified proteins from TABLE 1 are grouped according to their function and/or the specific protein complexes they belong to.

1. MOTOR PROTEINS

Protein	No. of peptides	Function
KIF11	0-25	Motor protein
KIF7	0-30	Motor protein
KIF14	0-28	Motor protein
KIF5B	0-20	Motor protein
KIF2A	0-13	Motor protein

3. CENTROSOMAL PROTEINS

Protein	No. of peptides	Function
CEP170	0-7	?
CEP192	0-18	Mitosis

5. KNOWN AURA INTERACTORS

Protein	No. of peptides	Function
TPX2	0-24	Mitosis
PLK1	0-6	Mitosis
KIF11	0-25	Mitosis
ARHGEF2	0-27	Mitosis
CEP192	0-18	Mitosis

2. CHROMATIN-REMODELING COMPLEXES

Protein	No. of peptides	Complex	Function
SMARCA4	0-35	Chromatin remodel.	ATP-dep. helicase
SMARCC1	0-35	SWI/SNF	Stimulator of ATPase activity
SMARCC2	0-15	SWI/SNF	Stimulator of ATPase activity
PBRM1	0-21	Chromatin remodel.	?
TOP2B	0-15	Chromatin remodel.	Topo-isomerase
ARID1A/B	0-23/0-10	Chromatin remodel.	Transcription regulation

4. DNA DAMAGE SIGNALING AND REPAIR

Protein	No. of peptides	Function
TP53-BP1	0-11	DDR
XPF/ERCC4	0-9	DDR
UPF1	0-7	DDR
TRIM28/KAP1	0-7	DDR
RAD50	0-7	DDR
ATRIP	0-9	DDR
WRN	0-8	DDR

6. SPLICEOSOME COMPLEX PROTEINS

Protein	Complex	No. of peptides	Function
SON	Spliceosome	0-34	Mitosis
SYF1	Spliceosome	0-6	TCR/ Transcriptional regulation
CDC5L	Spliceosome	0-6	Transcriptional regulation
SF3B2/3	Spliceosome	0-11/0-19	Pre-mRNA splicing
SLU7	Spliceosome	0-11	Pre-mRNA splicing
SFRS14	Spliceosome	0-12	Putative splicing factor

7. KNOWN MITOTIC PROTEINS

Protein	No. of peptides	Function
CENPF	0-51	Mitosis
ANILLIN	0-5	Cytokinesis
BUB1	0-7	SAC
SAS6	0-6	Mitosis
NEK1	0-15	Mitosis

8. MISCELLANEOUS

Protein	No. of peptides	Function
CPSF1/2	0-17/0-11	mRNA polyadenylation
CSTF3	0-14	mRNA polyadenylation
FIP1L1	0-7	mRNA polyadenylation
CSTF2T	0-5	?
APC (adenomatous polyposis coli)	0-13	Tumor suppressor

TABLE 1

* List of proteins identified by the mass-spectrometric approach, filtered using number of unique peptides.

* TPX2, Plk1 and KIF11 proteins served as a positive control for the list obtained.

IDENTIFIED PROTEINS	ACCESSION NO.	Mw (kDa)	CONTROL	SAMPLE
SWI/SNF complex subunit (SMARCC1)	Q92922	123	0	35
Centromere protein F (CENPF)	P49454	368	0	51
Isoform Short of Probable ubiquitin carboxyl-terminal hydrolase FAF-X (USP9X)	Q93008-1	290	0	41
Isoform D of Protein SON	P18583-5	267	0	34
SMARCA4 isoform 3	B1A8Z4	181	0	35
Isoform 1 of General transcription factor II-I (GTF2I)	P78347-1	112	0	33
Kinesin-like protein KIF7	Q2M1P5	151	0	30
Isoform 1 of Nuclear receptor coactivator 3 (NCOA3)	Q9Y6Q9-1	155	0	27
Isoform 1 of Ataxin-2-like protein (ATXN2L)	Q8WWM7-1	113	0	24
Kinesin-like protein KIF14	Q15058	186	0	28
Isoform 1 of Rho guanine nucleotide exchange factor 2 (ARHGEF2)	Q92974-1	112	0	27
"Isoform 1 of Arf-GAP with GTPase ANK repeat and PH domain-containing protein 3 (AGAP3)"	Q96P47-1	95	0	17
E3 ubiquitin-protein ligase UBR5 (UBR5)	O95071	309	0	32
Isoform 1 of Melanoma inhibitory activity protein 3 (MIA3)	Q5JRA6-1	214	0	27
Lysine-specific demethylase 6A (KDM6A)	O15550	154	0	24
Kinesin-like protein KIF11	P52732	119	0	25
Isoform 1 of Splicing factor 3B subunit 3 (SF3B3)	Q15393-1	136	0	19
Isoform 1 of AT-rich interactive domain-containing protein 1A (ARID1A)	O14497-1	242	0	23
Isoform 1 of SWI/SNF complex subunit SMARCC2	Q8TAQ2-1	133	0	15
Isoform 1 of Large proline-rich protein BAT3	P46379-1	119	0	25
Isoform 1a of Oxysterol-binding protein-related protein 3 (OSBPL3)	Q9H4L5-1	101	0	17
Isoform 1 of Protein transport protein Sec16A	O15027-1	234	0	24
Histone acetyltransferase p300	Q09472	264	0	24
Targeting protein for Xklp2 (TPX2)	Q9ULW0	86	0	24
Isoform 1 of Nuclear pore complex protein Nup214	P35658-1	214	0	15
Nuclear fragile X mental retardation-interacting protein 2 (NUFIP2)	Q7Z417	76	0	18
Kinesin-1 heavy chain KIF5B	P33176	110	0	20
Isoform 4 of Plectin-1 (PLEC1)	Q15149-4	516	0	18
Isoform 1 of Serine/threonine-protein kinase Nek1	Q96PY6-1	143	0	15

Isoform 1 of Protein polybromo-1 (PBRM1)	Q86U86-1	193	0	21
N-acetyltransferase 10 (NAT10)	Q9H0A0	116	0	15
"Isoleucyl-tRNA synthetase cytoplasmic (IARS)"	P41252	145	0	15
Isoform 1 of Lysine-specific histone demethylase 1(KDM1)	O60341-1	93	0	17
Activity-dependent neuroprotector homeobox protein (ADNP)	Q9H2P0	124	0	16
Isoform 1 of Serine/arginine repetitive matrix protein 2 (SRRM2)	Q9UQ35-1	300	0	17
"cDNA FLJ76788 highly similar to Homo sapiens splicing factor 3b subunit 2 145kDa (SF3B2)"	A8K485	100	0	11
WD repeat and HMG-box DNA-binding protein 1 (WDHD1)	O75717	126	0	13
Isoform 1 of Centrosomal protein of 192 kDa	Q8TEP8-1	213	0	18
Isoform Beta-2 of DNA topoisomerase 2-beta (TOP2B)	Q02880-1	183	0	15
Cleavage and polyadenylation specificity factor subunit 1 (CPSF1)	Q10570	161	0	17
Cytoplasmic dynein 1 heavy chain 1 (DYNC1H1)	Q14204	532	0	11
Isoform 1 of Chromodomain-helicase-DNA-binding protein 4 (CHD4)	Q14839-1	218	0	15
Pre-mRNA-splicing factor SLU7	O95391	68	0	11
Isoform 3 of Homeobox protein cut-like 1 (CUX1)	P39880-3	166	0	17
WD repeat-containing protein 33 (WDR33)	Q9C0J8	146	0	12
Zinc finger protein 295 (ZNF295)	Q9ULJ3	119	0	11
Solute carrier family 4 sodium bicarbonate cotransporter member 7 (SLC4A7)	B2CI53	127	0	10
Isoform 1 of Guanine nucleotide-binding protein-like 3 (GNL3)	Q9BVP2-1	62	0	10
Probable ATP-dependent RNA helicase DDX20	Q9UHI6	92	0	15
Component of gems 4 (GEMIN4)	P57678	120	0	15
Isoform 1 of Genetic suppressor element 1 (GSE1)	Q14687-1	136	0	10
Isoform 1 of AT-rich interactive domain-containing protein 1B (ARID1B)	Q8NFD5-1	236	0	10
Isoform 1 of WD repeat-containing protein C2orf44	Q9H6R7-1	79	0	9
Nucleolar protein 56 (NOP56)	O00567	66	0	6
Ribonucleases P/MRP protein subunit POP1	Q99575	115	0	12
Ubiquitin carboxyl-terminal hydrolase 10 (USP10)	Q14694	87	0	10
Isoform 1 of Tumor suppressor p53-binding protein 1 (TP53BP1)	Q12888-1	214	0	11
Isoform 2 of CAP-Gly domain-containing linker protein 1 (CLIP1)	P30622-1	161	0	7
6-phosphofructokinase type C (PFKP)	Q01813	86	0	12
Trophinin-associated protein (TROAP)	Q12815	84	0	8
Isoform 1 of Spermatogenesis-associated protein 5 (SPATA5)	Q8NB90-1	98	0	10
Isoform 1 of Interleukin enhancer-binding	Q12906-1	95	0	5

factor 3 (ILF3)				
Cleavage stimulation factor 77 kDa subunit (CSTF3)	Q12996	83	0	14
NF-kappa-B-repressing factor (NKRF)	O15226	78	0	7
Isoform 1 of Regulator of nonsense transcripts 1 (UPF1)	Q92900-1	124	0	7
Isoform 2 of R3H domain-containing protein 1 (R3HDM1)	Q15032-2	108	0	13
Isoform 1 of PAX-interacting protein 1 (PAXIP1)	Q6ZW49-1	118	0	15
CTTNBP2 N-terminal-like protein (CTTNBP2NL)	Q9P2B4	70	0	15
"Isoform 1 of Arf-GAP with GTPase ANK repeat and PH domain-containing protein 1 (AGAP1)"	Q9UPQ3-1	94	0	9
Isoform 1 of U2-associated protein SR140	O15042-1	118	0	10
Isoform Long of Adenomatous polyposis coli protein (APC)	P25054-1	312	0	13
Isoform 1 of PHD finger protein 3 (PHF3)	Q92576-1	229	0	9
Peroxisomal multifunctional enzyme type 2 (HSD17B4)	P51659	80	0	5
"Isoform 1 of Putative splicing factor arginine/serine-rich 14 (SFRS14)"	Q8IX01-1	120	0	12
Isoform 1 of WD repeat-containing protein 91 (WDR91)	A4D1P6-1	83	0	13
Isoform 2 of Kinesin-like protein KIF2A	O00139-2	75	0	13
Isoform 1 of Mediator of RNA polymerase II transcription subunit 23 (MED23)	Q9ULK4-1	156	0	13
Nucleolar GTP-binding protein 2 (GNL2)	Q13823	84	0	8
Isoform 1 of CCR4-NOT transcription complex subunit 1 (CNOT1)	A5YKK6-1	267	0	8
Serine/threonine-protein kinase PRP4 homolog (PRPF4B)	Q13523	117	0	8
Isoform 1 of 182 kDa tankyrase-1-binding protein (TNKS1BP1)	Q9C0C2-1	182	0	11
Cleavage and polyadenylation specificity factor subunit 2 (CPSF2)	Q9P2I0	88	0	11
Isoform 1 of DNA replication licensing factor MCM7	P33993-1	81	0	7
Isoform 1 of Transcription intermediary factor 1-beta (TRIM28)	Q13263-1	89	0	7
"cDNA FLJ53884 highly similar to mRNA decapping enzyme 1A (EC 3.-.-.-)"	B4DHN9	59	0	8
Nucleoprotein TPR	P12270	267	0	8
Isoform 1 of Angiomotin-like protein 1 (AMOTL1)	Q8IY63-1	107	0	7
ATP-dependent RNA helicase DDX1 OS=Homo sapiens GN=DDX1	Q92499	82	0	5
Isoform 1 of Zinc finger CCCH-type antiviral protein 1 OS=Homo sapiens GN=ZC3HAV1	Q7Z2W4-1	101	0	8
Nuclear pore complex protein Nup88	Q99567	84	0	9
Nuclear receptor coactivator 6 (NCOA6)	Q14686	219	0	7
Isoform 1 of Round spermatid basic protein 1-like protein (RSBN1L)	Q6PCB5-1	94	0	7
Isoform 1 of ATR-interacting protein (ATRIP)	Q8WXE1-1	86	0	9
Isoform 1 of Chromodomain-helicase-	Q9P2D1-1	336	0	9

DNA-binding protein 7 (CHD7)				
Cell division cycle 5-like protein (CDC5L)	Q99459	92	0	6
116 kDa U5 small nuclear ribonucleoprotein component (EFTUD2)	Q15029	109	0	6
Isoform 1 of DNA repair protein RAD50	Q92878-1	154	0	7
Werner syndrome ATP-dependent helicase	Q14191	163	0	8
Isoform 1 of CCR4-NOT transcription complex subunit 3 (CNOT3)	O75175-1	82	0	7
Isoform 1 of Hyaluronan mediated motility receptor (HMMR)	O75330-1	84	0	8
Isoform Long of Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A)	Q13627-1	86	0	6
Isoform 2 of Ubiquitin-associated protein 2-like (UBAP2L)	Q14157-1	104	0	9
DNA repair endonuclease XPF (ERCC4)	Q92889	104	0	9
Paired amphipathic helix protein Sin3a	Q96ST3	145	0	7
Isoform 1 of Actin-binding protein anillin (ANLN)	Q9NQW6-1	124	0	5
Isoform 1 of S phase cyclin A-associated protein in the endoplasmic reticulum (SCAPER)	Q9BY12-1	158	0	6
Probable E3 ubiquitin-protein ligase HERC2	O95714	527	0	6
Zinc finger CCCH domain-containing protein 11A (ZC3H11A)	O75152	89	0	5
"Isoform Alpha of Lamina-associated polypeptide 2 isoform alpha (TMPO)"	P42166-1	75	0	9
Isoform 1 of Codanin-1 (CDAN1)	Q8IWY9-1	134	0	7
Isoform 1 of Telomere-associated protein RIF1	Q5UIP0-1	274	0	6
"Isoform 1 of Proline-glutamic acid- and leucine-rich protein 1 (PELP1)"	Q8IZL8-1	120	0	5
Isoform 1 of Replication factor C subunit 1 (RFC1)	P35251-1	128	0	6
Pre-mRNA-splicing factor SYF1 (XAB2)	Q9HCS7	100	0	6
Isoform 1 of Histone-lysine N-methyltransferase MLL2	O14686-1	564	0	5
Isoform 1 of Probable E3 ubiquitin-protein ligase MYCBP2	O75592-1	510	0	7
Isoform 1 of ZZ-type zinc finger-containing protein 3 (ZZZ3)	Q8IYH5-1	102	0	10
Mastermind-like protein 1 (MAML1)	Q92585	108	0	7
Isoform 1 of Protein LAP2 (ERBB2IP)	Q96RT1-1	158	0	5
Isoform 2 of LisH domain and HEAT repeat-containing protein KIAA1468	Q9P260-2	139	0	8
Isoform 1 of Zinc finger MIZ domain-containing protein 1 (ZMIZ1)	Q9ULJ6-1	115	0	5
Isoform 1 of Protein FAM83D	Q9H4H8-1	64	0	5
Isoform 1 of TBC1 domain family member 4 (TBC1D4)	O60343-1	147	0	5
CCAAT/enhancer-binding protein zeta (CEBPZ)	Q03701	121	0	5
Isoform 1 of Protein FAM83D	Q9H4H8-1	64	0	5
Isoform 1 of TBC1 domain family member 4 (TBC1D4)	O60343-1	147	0	5
Isoform 1 of 5'-3' exoribonuclease 2 (XRN2)	Q9H0D6-1	109	0	6

Probable ATP-dependent RNA helicase YTHDC2	Q9H6S0	160	0	5
TOX high mobility group box family member 4 (TOX4)	O94842	66	0	6
Serine/threonine-protein kinase PLK1	P53350	68	0	6
Isoform 1 of Retinoblastoma-binding protein 5 (RBBP5)	Q15291-1	59	0	7
Activating signal cointegrator 1 (TRIP4)	Q15650	66	0	7
Isoform 1 of Centrosomal protein of 170 kDa	Q5SW79-1	175	0	7
Isoform 1 of Pre-mRNA 3'-end-processing factor FIP1 (FIP1L1)	Q6UN15-1	67	0	7
Semaphorin-4C	Q9C0C4	93	0	6
Isoform 1 of CCR4-NOT transcription complex subunit 10 (CNOT10)	Q9H9A5-1	82	0	5
Epidermal growth factor receptor substrate 15-like 1 (EPS15L1)	Q9UBC2	94	0	6
Calcium-binding mitochondrial carrier protein Aralar2 (SLC25A13)	Q9UJS0	74	0	6
Isoform 1 of Spermatogenesis-associated protein 5-like protein 1 (SPATA5L1)	Q9BVQ7-1	81	0	6
"Isoform 1 of 6-phosphofructokinase liver type (PFKL) "	P08237-1	85	0	5
"Isoform 1 of 6-phosphofructokinase muscle type (PFKM)"	P17858-1	85	0	6
Stromal interaction molecule 1 (STIM1)	Q13586	77	0	5
Probable ATP-dependent RNA helicase DHX37	Q8IY37	130	0	6
Apoptosis-stimulating of p53 protein 1 (PPP1R13B)	Q96KQ4	120	0	6
Isoform 1 of Activating signal cointegrator 1 complex subunit 2 (ASCC2)	Q9H1I8-1	86	0	6
YEATS domain-containing protein 2 (YEATS2)	Q9ULM3	151	0	6
Isoform 1 of C-jun-amino-terminal kinase-interacting protein 3 (MAPK8IP3)	Q9UPT6-1	147	0	7
Isoform 1 of La-related protein 7 (LARP7)	Q4G0J3-1	67	0	5
Isoform 1 of Coatomer subunit alpha (COPA)	P53621-1	138	0	5
Isoform RF1/RF2 of Retrotransposon-derived protein PEG10	Q86TG7-1	80	0	5
Mediator of RNA polymerase II transcription subunit 14 (MED14)	O60244	161	0	5
Isoform 1 of Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	O60566-1	120	0	7
Isoform Long of Trifunctional purine biosynthetic protein adenosine-3 (GART)	P22102-1	108	0	5
Spindle assembly abnormal protein 6 homolog (SASS6)	Q6UVJ0	74	0	6
Intron-binding protein aquarius (AQR)	O60306	171	0	6
Isoform 1 of Striatin (STRN)	O43815-1	86	0	5
Mediator of RNA polymerase II transcription subunit 24 (MED24)	O75448	110	0	5
Ubiquitin carboxyl-terminal hydrolase BAP1	Q92560	80	0	5
"Cleavage stimulation factor 64 kDa subunit tau variant (CSTF2T)"	Q9H0L4	64	0	5

TABLE 2

List of important MITOTIC proteins identified by the mass-spectrometric approach, filtered using number of unique peptides.

IDENTIFIED PROTEINS	ACCESSION NO.	Mw (kDa)	CONTROL	SAMPLE
Kinesin-like protein KIF7	Q2M1P5	151	0	30
Kinesin-like protein KIF14	Q15058	186	0	28
Kinesin-1 heavy chain KIF5B	P33176	110	0	20
Isoform 2 of Kinesin-like protein KIF2A	O00139-2	75	0	13
Kinesin-like protein KIF11	P52732	119	0	25
Isoform 1 of Centrosomal protein of 192 kDa	Q8TEP8-1	213	0	18
Isoform 1 of Centrosomal protein of 170 kDa	Q5SW79-1	175	0	7
Isoform 1 of Rho guanine nucleotide exchange factor 2 (ARHGEF2)	Q92974-1	112	0	27
Targeting protein for Xklp2 (TPX2)	Q9ULW0	86	0	24
Isoform D of Protein SON	P18583-5	267	0	34
Serine/threonine-protein kinase PLK1	P53350	68	0	6
Spindle assembly abnormal protein 6 homolog (SASS6)	Q6UVJ0	74	0	6
Isoform 1 of Actin-binding protein anillin (ANLN)	Q9NQW6-1	124	0	5
Cell division cycle 5-like protein (CDC5L)	Q99459	92	0	6
Isoform 1 of Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	O60566-1	120	0	7

TABLE 3

* List of proteins obtained from the 1st mass-spectrometric experiment, filtered using number of unique peptides.

* TPX2 and chTOG proteins served as a positive control for the list obtained.

IDENTIFIED PROTEINS	ACCESSION NO.	Mw (kDa)	CONTROL	SAMPLE
U5 small nuclear ribonucleoprotein 200 kDa helicase (EC 3.6.1.-)	U520	245	0	38
Supervillin (Archvillin) (p205/p250)	SVIL	248	0	23
RRP5 protein homolog (Programmed cell death protein 11)	RRP5	209	0	21
EIF4G1 variant protein (Fragment)	Q4LE58	178	0	23
SMARCA4 isoform 2	Q9HBD4	188	0	18
Cytoskeleton-associated protein 5 (Colonic and hepatic tumor over-expressed protein) (Ch-TOG protein)	CKAP5	226	0	16
CAD protein [Includes: Glutamine-dependent carbamoyl-phosphate synthase (EC 6.3.5.5); Aspartate carbamoyltransferase (EC 2.1.3.2); Dihydroorotase (EC 3.5.2.3)]	PYR1	243	0	16
"Hepatocellular carcinoma-associated antigen 90 (TPX2 microtubule-associated homolog) (Xenopus laevis)"	Q96RR5	89	0	17
HEAT repeat-containing protein 1 (Protein BAP28)	HEAT1	242	0	15
Exportin-7 (Exp7) (Ran-binding protein 16)	XPO7	124	0	18
Paired amphipathic helix protein Sin3a	SIN3A	145	0	15
Protein AATF (Apoptosis-antagonizing transcription factor)	AATF	63	0	15
Nuclear valosin-containing protein-like (Nuclear VCP-like protein)	NVL	95	0	14
ATP-dependent RNA helicase DHX8 (EC 3.6.1.-) (DEAH box protein 8) (RNA helicase HRH1)	DHX8	139	0	14
Hypothetical protein DKFZp686E0722 (Fragment)	Q68DX7	239	0	15
DNA (cytosine-5)-methyltransferase 1 (EC 2.1.1.37) (Dnmt1) (DNA methyltransferase HsaI) (DNA MTase HsaI) (MCMT) (M.HsaI)	DNMT1	183	0	10
Activity-dependent neuroprotector (Activity-dependent neuroprotective protein)	ADNP	124	0	16
Nardilysin precursor (EC 3.4.24.61) (N-arginine dibasic convertase) (NRD convertase) (NRD-C)	NRDC	132	0	8
Protein KIAA1429	Q69YN4	202	0	11
Chromodomain-helicase-DNA-binding protein 1 (EC 3.6.1.-) (ATP-dependent helicase CHD1) (CHD-1)	CHD1	197	0	9
Exportin-4 (Exp4)	XPO4	130	0	11
Nuclear pore complex protein Nup205 (Nucleoporin Nup205)	NU205	228	0	7
HBS1-like protein (ERFS)	HBS1L	75	0	10
LIM domain only protein 7 (LOMP) (F-box only protein 20)	LMO7	193	0	9

"Phospholipase C gamma 1"	A2A284	149	0	11
Actin-binding protein anillin	ANLN	124	0	10
Coiled-coil domain-containing protein 9	CCDC9	60	0	6
Hypothetical protein DKFZp434K1531	Q8ND04	110	0	7
Elongation factor Tu GTP-binding domain-containing protein 1	Q7Z2Z2	120	0	7
Kinesin-like protein KIF20A (Rabkinesin-6) (Rab6-interacting kinesin-like protein) (GG10_2)	KI20A	100	0	9
RNA polymerase-associated protein CTR9 homolog (SH2 domain-binding protein 1)	CTR9	134	0	5
Pre-mRNA-processing-splicing factor 8 (Splicing factor Prp8) (PRP8 homolog) (220 kDa U5 snRNP-specific protein) (p220)	PRP8	274	0	8
MutS homolog 3 (E. coli)	A1L480	128	0	6
"Presequence protease mitochondrial precursor (EC 3.4.24.-) (hPreP) (Pitrilysin metalloproteinase 1) (Metalloproteinase 1) (hMP1)"	PREP	117	0	6
Novel protein	Q5JXI5	95	0	9
Nucleolar protein 9	NOL9	79	0	8
Digestive organ expansion factor homolog	DEF	87	0	8
DNA ligase 3 (EC 6.5.1.1) (DNA ligase III) (Polydeoxyribonucleotide synthase [ATP] 3)	DNL3	103	0	7
Probable ATP-dependent RNA helicase DDX31 (EC 3.6.1.-) (DEAD box protein 31) (Helicain)	DDX31	94	0	7
Uncharacterized protein KIAA1033	K1033	136	0	7
Periodic tryptophan protein 2 homolog	PWP2	102	0	6
Dedicator of cytokinesis protein 7	DOCK7	243	0	6
"Neurolysin mitochondrial precursor (EC 3.4.24.16) (Neurotensin endopeptidase) (Mitochondrial oligopeptidase M) (Microsomal endopeptidase) (MEP)"	NEUL	81	0	5
Rab GTPase-binding effector protein 1 (Rabaptin-5) (Rabaptin-5alpha) (Rabaptin-4) (Renal carcinoma antigen NY-REN-17)	RABE1	99	0	5
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1 (EC 3.6.1.-) (ATP-dependent helicase 1) (hHEL1)	SMRCD	117	0	6
TFIIH basal transcription factor complex helicase subunit (EC 3.6.1.-) (DNA-repair protein complementing XP-D cells) (Xeroderma pigmentosum group D-complementing protein) (CXPB) (DNA excision repair protein ERCC-2)	ERCC2	87	0	6
Zinc finger CCHC domain-containing protein 8	ZCHC8	79	0	5
Glutamine-rich protein 1	QRIC1	86	0	5
Tyrosine-protein kinase-like 7 precursor (Colon carcinoma kinase 4) (CCK-4)	PTK7	118	0	5
mitochondrial precursor (IF-2Mt) (IF2(mt)) (IF-2(Mt))"	IF2M	81	0	5
Aspartyl/asparaginyl beta-hydroxylase (EC 1.14.11.16)	ASPH	86	0	6
ATR-interacting protein (ATM and Rad3-	ATRIP	86	0	6

related-interacting protein)				
Hepatocyte growth factor-regulated tyrosine kinase substrate (Protein pp110) (Hrs)	HGS	86	0	6
Protein bicaudal D homolog 2 (Bic-D 2)	BICD2	94	0	6
Zinc finger protein 574	ZN574	99	0	5
Exocyst complex component 8 (Exocyst complex 84 kDa subunit)	EXOC8	82	0	5
Afadin (Protein AF-6)	AFAD	206	0	6
Transcription termination factor 1 (TTF-1) (TTF-I) (RNA polymerase I termination factor)	TTF1	101	0	5
Ubiquitin carboxyl-terminal hydrolase 36 (EC 3.1.2.15) (Ubiquitin thioesterase 36) (Ubiquitin-specific-processing protease 36) (Deubiquitinating enzyme 36)	UBP36	123	0	5
Tetratricopeptide repeat protein 7B (TPR repeat protein 7B) (Tetratricopeptide repeat protein 7-like-1)	TTC7B	83	0	5
RNA polymerase II-associated protein 1	RPAP1	153	0	5
"X-prolyl aminopeptidase (Aminopeptidase P) 1 soluble"	Q5T6H7	62	0	5
CLIP1 protein	A0AVD3)	162	0	6
Coronin-7 (70 kDa WD repeat tumor rejection antigen homolog)	CORO7	101	0	5
CTTNBP2 N-terminal-like protein	CT2NL	70	0	5
NEDD8 ultimate buster 1 (Negative regulator of ubiquitin-like proteins 1) (Renal carcinoma antigen NY-REN-18)	NUB1	71	0	5
"A kinase anchor protein 1 mitochondrial precursor (Protein kinase A-anchoring protein 1) (PRKA1)"	AKAP1	97	0	5
Long-chain-fatty-acid--CoA ligase 4 (EC 6.2.1.3) (Long-chain acyl-CoA synthetase 4) (LACS 4)	ACSL4	79	0	5
IWS1 homolog (IWS1-like protein)	IWS1	92	0	5

TABLE 4

List of proteins found common in both MS-experiments.

IDENTIFIED PROTEINS	ACCESSION NO.	Mw (kDa)
SMARCA4 isoform 3	B1A8Z4	181
Targeting protein for Xklp2 (TPX2)	Q9ULW0	86
Isoform 1 of Actin-binding protein anillin (ANLN)	Q9NQW6-1	124
Paired amphipathic helix protein Sin3A	Q96ST3	145
Cell division cycle 5-like protein (CDC5L)	Q99459	92
Isoform 1 of Protein polybromo-1 (PBRM1)	Q86U86-1	193
Kinesin-like protein KIF11	P52732	119

CURRICULUM VITAE

PERSONAL DETAILS

Name: Payal Bhatia
Date of Birth: 28 Sept. 1983
Sex: Female
Nationality: Indian

EDUCATION

PhD, Cancer Biology, University of Zurich, Switzerland- Since December 2006

I joined the Institute of Molecular Cancer Research as a graduate student under the supervision of Dr. PD. Stefano Ferrari. The objective of my project is to understand the effect of DNA damage on a mitotic kinase Aurora A and on progression of cells through mitosis. I will be finishing my PhD in 2010.

Masters in Science (M.Sc), Plant Molecular Biology, University of Delhi, India- 2004-2006

This 2-year intensive course included a detailed theoretical and practical training in basic concepts of molecular biology, using plant as the model system. I was 2nd among the 6 students admitted through a nationwide selection examination followed by interviews.

Dissertation

In addition to the above-mentioned courses, I completed a dissertation entitled '**Analysis of Auxin-Inducible genes (*OsIAA4* and *OsIAA10*) of rice by raising overexpression lines in *Arabidopsis thailiana***' towards my Master thesis.

Bachelors in Science (B.Sc), Microbiology, University of Delhi, India- 2001-2004

I obtained my Bachelors degree in Microbiology that included 3-years of detailed theoretical and practical training in various aspects of biology.

SCIENTIFIC EXPERIENCE & PROJECTS

As a dissertation for my Master course, I carried out a 1-year project under the supervision of Prof. J.P.Khurana at the University of Delhi, India. The project was entitled '**Functional Analysis of Auxin-Inducible genes (*OsIAA4* and *OsIAA10*) of rice by raising overexpression lines in *Arabidopsis thailiana***'. Through this project I intended to functionally characterize two of the rice *Aux/IAA* genes, *OsIAA4* and *OsIAA10* by studying their overexpression in *Arabidopsis*.

The starting point was to raise overexpression lines of *OsIAA4* and *OsIAA10* in *Arabidopsis*. This was done by performing *Agrobacterium*-mediated transformation of *Arabidopsis* using floral-dip method and the presence of transgenes was confirmed by tissue PCR. The aim was to observe any phenotypic and/or physiological effects

during plant growth arising due to overexpression of these genes. Root elongation assay was performed to determine changes in root growth and development. Morphological changes, for example, changes in the pattern of leaf development were also scored. In addition, sensitivity of overexpressing lines towards different auxin concentrations was tested. This was related to the extent of expression of these genes as determined by RT-PCR analysis. Also, I determined the level of expression of these genes in different plant organs such as roots, shoots, flower and coleoptiles to better understand their effect on plant development.

Second part of the project involved production of recombinant protein in bacteria to study the stability of OsIAA4 and OsIAA10 proteins. For this, stability of these proteins in varying concentrations of auxin was determined and related with the absence or presence of domain II in the protein that is responsible for promoting degradation.

ACADEMIC ACHIEVEMENTS AND SCHOLARSHIPS

- ✦ I was selected for my graduate studies by the University of Zurich through a worldwide selection procedure.
- ✦ I was awarded a **University Gold Medal** by the University of Delhi in 2007 for Plant Molecular Biology course examinations.
- ✦ I was granted with **Monsanto Scholarship** for my Masters coursework. The basis of selection was excellent performance in written and oral examination held for entering the program and 1st year academic performance.
- ✦ I was awarded a **University Gold Medal** by the University of Delhi in 2005 for Microbiology course examinations.
- ✦ I was entitled the **Best Student of Microbiology** by the institution for the year 2004.
- ✦ I was awarded with a number of cash prizes throughout my bachelor's coursework for consistent academic performance.

PUBLICATIONS

Payal Bhatia, Mirco Menigatti, Michele Brocard, Simon Morley and Stefano Ferrari. 2010. Mitotic DNA damage targets the Aurora A-TPX2 complex. *Cell Cycle*, Nov15, 9(22), 4592-4599.

Book Chapter

Payal Bhatia and Stefano Ferrari. 2008. Role of cell cycle controllers in the development of cancer. Multiple pathways in Cancer Development, 69-86, ISBN: 978-81-7895-362-5.

ORAL/POSTER PRESENTATIONS

2010

- ✦ Oral presentation, RRR (Replication, Repair and Recombination) club, Cancer Biology, Zurich, Switzerland, April 2010. Mechanism of regulation of Aurora A kinase in response to mitotic DNA damage. **Payal Bhatia** and Stefano Ferrari.

2009

- ✦ Oral presentation, Cancer Biology Students' Retreat, Bogis-Bossey, Switzerland April 2009. Mechanism of regulation of Aurora A kinase in response to DNA damage. **Payal Bhatia** and Stefano Ferrari.

2008

- ✦ Poster presentation, Cancer Biology Retreat, Fiesch, Switzerland, January 2008. Mechanisms of regulation of a mitotic kinase, Aurora A, upon DNA damage. **Payal Bhatia**, Agnieszka Krystyniak and Stefano Ferrari.
- ✦ Poster presentation, International Cell Cycle and Cancer Conference, Toulouse, France, March 2008. Mechanisms of regulation of a mitotic kinase, Aurora A, upon DNA damage. **Payal Bhatia**, Agnieszka Krystyniak and Stefano Ferrari.

2007

- ✦ Poster presentation, Cancer Immunosurveillance and Immunotherapy, Charles Rodolphe Brupbacher Symposium, Zurich, Switzerland, March 2007. Mechanisms of regulation of a mitotic kinase, Aurora A, upon DNA damage. **Payal Bhatia**, Agnieszka Krystyniak and Stefano Ferrari.
- ✦ Poster presentation, Cancer Biology Students' Retreat, Eggberge, Switzerland, Sept. 2007. Mechanisms of regulation of a mitotic kinase, Aurora A, upon DNA damage. **Payal Bhatia**, Agnieszka Krystyniak and Stefano Ferrari.

LABORATORY SKILLS

Biochemistry/Cell and Molecular Biology

Isolation of DNA/RNA

Electrophoresis

Immunoblotting

Polymerase Chain Reaction (Real-Time, RT)

Cloning

In vitro pull down assays, Immunoprecipitation

Protein expression

Mammalian cell culture

Cell synchronization with different methods

Cell transfection (DNA, RNA, siRNA oligonucleotides)

Protein transduction

Preparation of whole cell extracts

Microtubule regrowth assay

Flow cytometry

Protein kinase assays

Silver staining, In-gel protein trypsinization

Microscopy

Immunofluorescence

Time lapse/Live-cell imaging

Analysis software known

ImageJ

Photoshop

Scaffold

Summit

CellR

Leica Application Suite

ADDITIONAL ACTIVITIES

♦ 2001-2004. **Secretary**, Microbiologika Society, Ram Lal Anand College, University of Delhi, India.

♦ Nov. 2008- April 2009. **Co-organizer**, 2nd Cancer Biology Students' Retreat, Cancer Biology PhD Program, University of Zurich and ETH, Switzerland.

♦ Oct. 2009. **Teachers Assistant**, Student course in Applied Molecular Cancer Research, University of Zurich, Switzerland.

REFERENCES

Available upon request.